

The Plight of Trees in Disturbed Forest: Conservation of Montane Trees, Nigeria

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Equations

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CHAPTER 1

Introduction

1.1 The Afromontane Archipelago

The montane regions of Africa are arguably one of Earth's most interesting terrestrial biomes: their geological history is dramatic and their biology diverse and fascinating. Though the African continent is largely a rather flat plateau, tectonic activity—volcanism, rifting and uplift—during the Miocene created isolated regions of mountain ranges (reviewed in Grimshaw 2001). These stretch from the Loma Mountains (Sierra Leone), east to the Red Sea Hills (Sudan) and the Ahl Mescat Hills (Somalia), and south toward the Cape Peninsula (White 1981). The isolated spatial structure of these mountains amidst a “sea” of lowland habitat lead White (1978, 1981) to analogize this system to islands in an archipelago; the “Afromontane archipelago” (FIGURE 1.1).

Part of the Afromontane archipelago's intrigue is that despite local areas containing many endemics (or near endemics) there is striking uniformity of vegetation composition across much of the system, which has lead to recognition of a specific phytochorion (a geographic region with a relatively uniform vegetation profile) for Afromontane regions (White 1981). This is fascinating, considering that mountains sharing similar species and flora assemblies can be separated by thousands of kilometers of inhospitable habitat (White 1981). *Metarungia pubinveria* is a prime exemplar: until 2008, this species had only been recorded in the montane regions of East Africa; its discovery in a single forest in the highlands of eastern Nigeria represented a range disjunction of some 1200km and the first, and so far, only record of this species and genus in West Africa (Darbyshire et al. 2008).

The patterns observed amongst Afromontane biota are a product of the geological and climatic history of Africa. Since ~800,000 years BP, the Earth has been cycling through a series of glacial and warming events, with a periodicity of ~100,000 years (Maley 1996). Periods of lower temperatures would have facilitated the extension of montane forest elements into the lowlands, which would have also assisted the movement of taxa between mountain ranges; during warming periods, these montane elements would then contract to refugia at higher altitudes (Maley 1996).

Studies utilizing fossilized pollen, wood and animals, and measurements of historical lake levels and sediments, concur that the climate and vegetation in Africa has seen dramatic fluctuations and major shifts in boundaries of montane forests (Hamilton and Taylor 1991, Elenga et al. 2000, Dupont et al. 2001).



FIGURE 1.1 | Approximate distribution of major montane habitats in Africa. The configuration of island-like montane areas creates a situation analogous to an archipelago.

Ultimately, this pattern of events has had a marked impact on the region's biota. As mentioned, it creates remarkable similarity between montane regions and impressive disjunctions, but the fragmentation of populations, in correlation to retracting habitat, also sets the stage for divergent evolution. The lack of interconnecting forest between mountains means gene flow among populations is arrested, driving genetic differentiation (McDonald and Daniels 2012, Kadu et al. 2013). However, in some cases it is possible that long-distance dispersal may occur, which modulates divergence patterns amongst populations (Griswold 1991). The expanding collection of systematic/cladistic studies in montane Africa demonstrate that patterns of divergence amongst species, by and large, reflect the patterns of isolation amongst Afromontane regions: for example, spiders (Griswold 1991), *Peripatopsis* velvet worms (McDonald and Daniels 2012), turacos (Njabo and Sorenson 2009), *Rhinolophus* bats (Taylor et al. 2012), and *Chlorocebus* monkeys (Tosi 2008). It has also been demonstrated that ancient dispersal events of temperate floral taxa into Africa have

survived in montane refugia, which have similar climatic conditions to temperate zones, and thus their descendants now currently contribute significantly to their community composition (Gehrke and Linder 2009).

The conservation importance of Afromontane habitats should be stressed, since these areas harbour relict populations, endemics, and often—when one takes the time to look—species new to science (White 1981, Kahindo et al. 2007, Darbyshire et al. 2008, Branch and Bayliss 2009, Taylor et al. 2012, McDonald and Daniels 2012). Kahindo et al. (2007) argue for a greater emphasis of genetics in conservation planning for Afromontane species because the identification of genetically distinct, but possibly cryptic, lineages has a number of points relevant to their protection: (1) the extent of biodiversity is not truly appreciated until phylogenetic analyses are conducted; (2) these analyses reveal greater local endemism; which (3) often means that distinct taxa exist in much smaller and isolated populations than may have been previously envisioned.

The future of Afromontane habitats is in a precarious position.

“The survival of Afromontane vegetation is an ongoing struggle between the daily necessities of poverty-stricken Africa, and the values perceived by those more fortunate.” ~ Grimshaw, 2001

As will be discussed in the following section, heavy anthropogenic pressure across Africa threatens biodiversity and ecosystem function, but Afromontane forests are perhaps exceptionally vulnerable due to their limited size and constraints in their range. Africa’s climate is expected to change dramatically by the end of this century: precipitation patterns are likely to change, temperatures could increase by as much as 5.1°C, and the rate of change will progressively increase (Willis et al. 2013). Modeled changes are shown to parallel those that have occurred in past, based on palaeoclimatic evidence, through which contemporary taxa were able to persist (Willis et al. 2013). However, while montane taxa have historically shown remarkable resilience to fluctuating climate through their environmental plasticity, human disturbance poses an unprecedented potential obstacle to possible future movements (Hamilton and Taylor 1991).

1.2 Conservation in Africa

The African biota suffers at the hand of humans; ongoing pressure from agriculture, fire, logging, hunting, extension of infrastructure, and global warming in general, threaten the diversity, characteristics and dynamics of ecological communities (Akani et al. 2001, Wethered and Lawes 2005, Adekunle 2006, Fa et al. 2006, Croes et al. 2007, Adekunle et al. 2010, Norris et al. 2010,

Kreyling et al. 2010, Ofori-Boateng et al. 2013, Gower et al. 2013, Hundera et al. 2013, Schmitt et al. 2013). Combining conservation and local community goals is seen as the most sustainable way to protect declining African forests (Brookini et al. 2012), and studies—in the Afrotropics and Neotropics—support the notion that agroforestry systems can provide a buffer against anthropogenic pressure by providing altered habitats that retain some of their original qualities (Cassano et al. 2012, Mulwa et al. 2012). While community conservation efforts are the ideal choice in the delicate balancing act between people and the environment, the desired objectives are often unobtainable in practice, and there are a large number of reasons for this. Causes of failure for community conservation in Africa, as outlined by Adams and Hulme (2001), can range from lack of resources and sustainability of the project, the inability of project managers to deliver on their promises, and unresolved conflicts with local people (amongst others).

Conservation of forest in Africa is also further limited by unequal distribution of research effort across the different geographic regions. Relative to other Afrotropical regions, the West African forests have poorly understood and described biodiversity (Norris et al. 2010), but are globally recognized as significant hotspots for biodiversity (Bergl et al. 2007, Norris et al. 2010, Brookini et al. 2012). The tropical forest landscape of West Africa is a changing face of once dense tree cover shifting towards degradation and fragmentation, as trees are felled to make way for agriculture (Norris et al. 2010). The increased presence of agroforest mosaics and modified habitat globally has led to the notion of an “Era of Secondary Forest” (Lugo 2009). Lugo (2009) argues that too much research and conservation effort has been invested into primary forests, which are seen as the pinnacle of community succession. Instead, she takes the view that these new, modified forest communities represent important ecological entities (which thrive in the presence of human presence and invasion of exotic species) and should be eligible for conservation.

Needless to say, the conservation of tropical forest in West Africa, but also Africa in general, provides conservationists with a real challenge. A challenge that requires a balance between meeting the needs of local people, determining importance of historical versus future ecosystems, and protecting biodiversity in the face of increasing degradation and disturbance.

1.3 Habitat Fragmentation and Disturbance

Fragmentation reduces the area of habitat available and ultimately leads to smaller population sizes. In addition, the breakup of large continuous forest into smaller “island” fragments (amidst a matrix of non-forest habitat) has the potential to create population disconnect. These changes to habitat and landscape may threaten species by negatively impacting their biology, behavior, ecology

and genetics (see Fischer and Lindenmayer 2007). This section will discuss these impacts, starting with an overview of the importance of genetics in conservation, and ending with a focus on trees in degraded forests.

1.3.1 Genetics in conservation

Genetic diversity is now understood to be a key factor in maintaining healthy populations of organisms (Frankham et al. 2011). Conservation genetics involves utilizing population genetic theory and techniques to minimize risks of extinction in threatened species (Frankham et al. 2011). A conservationist's use of molecular tools should provide them with insight into the patterns of genetic diversity in populations and the processes that shape this diversity—with the goal of maintaining this diversity and these processes. Maintaining genetic diversity in populations is of importance because it provides a substrate for evolution to occur (Frankham et al. 2011). In other words, the adaptation of organisms to environmental change requires a variable pool of different gene variants (alleles) in a population, such that said population is able to “respond” to selective pressures (Frankham et al. 2011). Characterization of genetic diversity in populations is done by assessing the variation of alleles at particular genes or loci (Frankham et al. 2011).

New alleles in populations are generated by mutation of the DNA and the frequencies of these alleles are fashioned by the processes of natural selection, migration (or gene flow), and genetic drift: these four mechanisms are the forces of evolution, which can be defined as a change in the genetic composition of a population (Frankham et al. 2011). While mutation is the only process that actually creates new alleles in populations, its effect on allele frequencies is minimal due to the slow rate at which mutations accrue naturally (Frankham et al. 2011). Mutations provide genetic novelty to populations, introducing new material that selection can act on. The adaptation and differentiation of populations to local conditions can be facilitated by the accumulation of beneficial mutations in the population (Shapiro et al. 2012), and the evolution of new species can be linked to the acquisition of new genes that provide novel ecological functions, leading to speciation (Wheat et al. 2007, Tellgren-Roth et al. 2009).

Genetic drift is a random process that reduces genetic diversity and drives divergence between populations (Frankham et al. 2011). Populations that reproduce sexually produce a successive generation derived from a sample of possible gene combinations from the parental generation (Frankham et al. 2011). In smaller populations, some alleles (particularly rare ones) risk the chance of becoming lost from the gene pool as a result of incomplete sampling, and are thus not transmitted to the progeny generation. Thus, drift is a random process that causes allele frequencies to fluctuate

from generation to generation (O'Hara 2005, Frankham et al. 2011). Founder events and bottlenecks also create drift because populations undergo ephemeral reductions in population size, which subsamples but a portion of alleles from the ancestral population (Clegg et al. 2002, Smith et al. 2012). Natural selection is also a force that generally drives divergence between populations by favoring alleles that provide the greatest fitness benefit to local conditions (Jump et al. 2006, Frankham et al. 2011, Shapiro et al. 2012). There are, however, examples where natural selection homogenizes populations (Benkman and Parchman 2013). The force of selection is weakened by smaller population size (Frankham et al. 2011).

Gene flow is a process that counteracts diverging processes of selection and drift, homogenizing allele frequencies amongst populations; it can also increase genetic variance in a population by bringing variance from external sources (Frankham et al. 2011). A classic example are the water snakes (*Natrix sipedon*) of Lake Erie studied by Camin and Ehrlich (1958); these researchers demonstrated that migration can oppose selection, resulting in more populations with more similar trait frequencies than expected under selection alone.

1.3.2 Genetic effects of fragmentation

From population genetic theory, we expect habitat fragmentation to lead to random fluctuations in allele frequencies within and between populations such that populations become more differentiated from one another—in other words, greater genetic structuring. This is because populations of smaller size sample more haphazardly from the available gene pool, which can lead to fixation of alleles (Charlesworth 2009). The scientific literature is full of case-studies illustrating increased spatial genetic structure and loss of within-population diversity with increasing fragmentation (Reed et al. 2009, Tracy and Jamieson 2011, Taylor et al. 2011, Rubidge et al. 2012, Breed et al. 2012). For example, comparisons of ancient and modern populations of yellowhead, *Mohousa ochrocephala*, demonstrate that increased fragmentation of habitat (associated with rising anthropogenic pressure) through time has resulted in greater differentiation amongst contemporary populations, relative to historical counterparts; modern populations were also found to have some 22.6% less allelic diversity than historical ones (Tracy and Jamieson 2011).

Another genetic consequence of fragmentation is a rise in inbreeding. Reduced population size should also correlate with increased inbreeding due to fewer potential mates and a greater probability of common descent amongst individuals (Frankham et al. 2011). Furthermore, the increased frequency of homozygosity in the population (as a consequence of inbreeding) is expected to depress the fitness of the population (“inbreeding depression”) as recessive deleterious

alleles become expressed or as fitter heterozygous allele combinations become less frequent (Keller and Waller 2002). These negative consequences of inbreeding have been found in plants and animals (Saccheri et al. 1998, Andersen et al. 2004, Jones and Comita 2008, Miller et al. 2012, Breed et al. 2012). However, if adequate dispersal amongst subpopulations can be maintained, negative genetic effects need not arise from fragmentation. For example, a *Formica lugubris* ant population studied by Gyllenstrand and Seppä (2003) was found to have high genetic diversity at both nuclear microsatellite and mitochondrial loci, despite apparent isolation and fragmentation of subpopulations. The inbreeding coefficient was small and non-significant, and authors concluded that male dispersal was considerable enough amongst subpopulations to resist drift, maintain high variation, and avoid mating amongst relatives.

Finally, small, fragmented populations, with lower genetic variance, may show evolutionary constraints—an inability to adapt to selection—and smaller populations in fragmented landscapes can show fitness declines (Wade et al. 1996, Johansson et al. 2007, Reed 2010, Ashworth and Martí 2011). Though genetics is undoubtedly an important part of a population or species' long-term viability, newer opinions promote avoiding the view of genetic effects in isolation, as environment, selection, life-histories and stochasticity will also play significant roles (Willi et al. 2006, Reed 2010).

1.3.3 Anthropogenic disturbance, forest fragmentation, and ecological impacts

Increasing landscape modification and land use intensity will break up and decrease native vegetation, which endangers the persistence of species (Fischer and Lindenmayer 2007). Habitat disturbances reduce habitat quality and the impact on organisms is linked to relative hostility of the matrix surrounding fragments (Gascon et al. 2000, Fischer and Lindenmayer 2007, Bracebridge et al. 2012, McConkey et al. 2012). The loss of biodiversity and abundances of species is often associated with habitat disturbance and fragmentation (Cosson et al. 1999, Lawes et al. 2005, Brandão and Araújo 2008, Phoonjampa et al. 2011, Thornton et al. 2012, Pardow and Lakatos 2013), though rodent communities have been found to do reasonably well in disturbed forest (Isabirye-Basuta and Kasenene 1987, Decher 1997), and generalist species tend to fare better than specialists (Kirika et al. 2008). While forest fragments do, however, have conservation value (Decher 1997), long-term conservation should take into consideration that disturbed forest habitat is not optimal habitat (Fischer and Lindenmayer 2007, Cassano et al. 2012). Furthermore, it is also imperative to acknowledge that forest degradation can lead to a possible extinction debt, in which

the effects of anthropogenic disturbance are not immediately felt but have ecological, genetic, and biodiversity consequences in the future (Kuussaari et al. 2009).

As continuous forest is broken up, forest edge and the distance between suitable habitat patches increases. Edge effects alter the microclimate in the forest, maybe as much as 300m into the forest interior (Gascon et al. 2000, Pardow and Lakatos 2013), and breaks in the forest can provide a significant hindrance to the movement and distribution of species (Laurance and Gomez 2005, Oliveira et al. 2011). The size constraints of suitable patches can limit the distribution of populations and alter their behavior in response to low quality resource availability (Irwin 2008, Jorge and Howe 2009, Savini et al. 2009). However, if individuals are able to cross the matrix between patches, the spatial disconnect amongst fragments can be trumped by their functional connectivity (Wieczkowski 2010). Species thus respond differently to fragmentation depending on their biology and ecological traits (Brotons et al. 2003, Baranga 2004, Cramer et al. 2007, Thornton et al. 2012) and their ability to utilize the surrounding matrix (Lehouck et al. 2009, Wieczkowski 2010), and this should be taken into account by conservation management.

1.3.4 Habitat degradation and tree populations: Regeneration and evolution

Anthropogenic disturbances can hamper natural recruitment processes in tree populations. Edge effects have been correlated to depressed regeneration and lower seedling abundances, possibly driven by such mechanisms as area-to-edge interactions, biotic sources of damage, and various altered microhabitat variables—e.g. hotter air and soil temperatures, lower humidity, more intense solar radiation and increased leaf litter accumulation (Bruna 1999, 2002, Didham and Lawton 1999, Benítez-Malvido and Martínez-Ramos 2003, Benítez-Malvido and Lemus-Albor 2005, Sugiyama and Peterson 2013).

Thus, human activity that increases the prevalence of edges poses a major conservation issue; however, work by Bouroncle and Finegan (2011) suggests that forests need not “melt down” at their edges provided they have functional characteristics that buffer from disturbance. Fire can create drastic changes in vegetation structure and may open up the canopy at edges, facilitating greater penetration of edge effects (Didham and Lawton 1999, Slik et al. 2011). It is incredibly dangerous to long-term regeneration of forest because it creates cycles of desiccation and fuel accumulation that increases a forest’s susceptibility to burning (Cochrane et al. 1999). A history of logging may also have negative effects on regeneration (Grogan and Galvão 2006, Vieira et al. 2007, Duclos et al. 2013), and introduced ungulates can also have negative impacts (Cole et al. 2012).

Unlike other organisms, trees are sessile, and the only long-distance movement that generally occurs is during the reproductive cycle in the stages of pollen flow and seed dispersal (Dick et al. 2008, Bacles and Jump 2011). The evolution of plant populations can be redirected by the effects of fragmentation, creating a cascading, cycling feedback loop between ecological traits, ecological interactions, and the underlying population genetic variance (Jacquemyn et al. 2012; FIGURE 1.2). Ultimately, fragmentation creates a new selection regime, and trees must evolve to meet the new requirements or have ecological interactions that allow them to cope in the altered environment (Eckert et al. 2010, Jacquemyn et al. 2012). For example, in a population of *Pachira quinata*, while a smaller percentage of flowers produced fruit in isolated trees (relative to those in continuous forest), the overall fruit production per tree was non-significantly different because isolated trees were buffered by greater flower production (Fuchs et al. 2003). Also, Dick et al. (2003) demonstrated that introduced Africanized honeybees (*Apis* sp.) were important in creating functional connectivity amongst forest fragments in populations of *Dinizia excelsa*, and actually helped to increase genetic neighbourhood size.

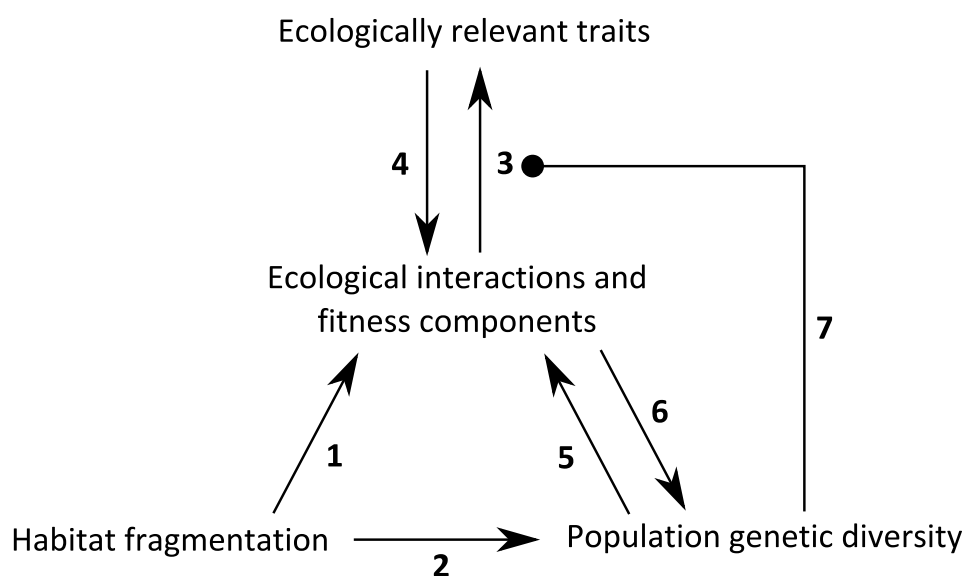


FIGURE 1.2 | Ecological change driven by fragmentation. Habitat fragmentation may alter ecological interactions and fitness (1) and through stochasticity decrease genetic diversity (2). Altered interactions may later manifest as new phenotypes with genetic basis (3) and this may then feedback to modulate ecology (4); however, the evolution of traits will depend on the genetic diversity available (7). Genetic diversity will impact population fitness and ecological interactions (5), but the reverse is also true (6). SOURCE: modified from Jacquemyn et al. (2012), with permission.

The general paradigm that populations lose genetic diversity with increasing fragmentation is controversial in plants due to contradicting conclusions amongst studies, and the maintenance of genetic variance in the population will be dependent on the mating system and the extent of gene movement. High levels of genetic diversity have been observed in fragmented tree populations, attributed to significant pollen flow or seed dispersal distances (Dick et al. 2003, Lourmas et al. 2007, Born et al. 2008, Fuchs and Hamrick 2011). However, evolutionary theory does predict that changes in mating systems will occur if habitat disturbance drives changes in the extent of pollen availability (Eckert et al. 2010). This change in turn will depend on the intensity at which pollen movement is impacted and/or the availability of conspecific mates is reduced (Eckert et al. 2010).

Quesada et al. (2013) found that progeny of *Ceiba aesculifolia* trees in disturbed habitat received but a fraction of the pollen donor diversity—relative to those in undisturbed habitat—which was attributed to constrained bat pollinator movement. Other studies support declines in pollinator efficiency in fragmented environments (Ghazoul et al. 1998, Aguilar and Galetto 2004, Fernández-M and Sork 2007, Ismail et al. 2012), and pollen limitation may hinder successful regeneration (Anderson et al. 2011). Shifts to a mating system with greater selfing rates (versus outcrossing), increased inbreeding and correlated paternity in progeny, and increasing differentiation amongst cohorts are also documented in cases where fragmentation has broken up populations and decreased conspecific adult density (Murawski and Hamrick 1991, Aldrich and Hamrick 1998, Dick et al. 2003, Fuchs et al. 2003, Ismail et al. 2012).

Dispersal of seed is important in mitigating the genetic effects of fragmentation and patterns in the seed rain can contribute to the population's genetic structuring (Heuertz et al. 2003, Born et al. 2008, Herrera and García 2009, Fuchs and Hamrick 2010, Collevatti and Hay 2011, Ismail et al. 2012). Seed dispersal is also a key process in the regeneration of trees, linking adult reproduction—pollination and fruit production—to processes that regulate establishment of progeny—i.e. survival of seed post-dispersal, seedling establishment and sapling growth (McConkey et al. 2012). Ergo, changes in natural dispersal phenomena could significantly impact regeneration. Fragmentation and hunting can deplete important seed disperser populations, which limits the removal and dispersal of seed, and may ultimately impact patterns of regeneration (Cordeiro and Howe 2001, Babweteera et al. 2007, Kirika et al. 2008, Babweteera and Brown 2010, Chimera and Drake 2010, Collevatti and Hay 2011, Effiom et al. 2013). Fragmentation may also shift the degree to which granivores act as mutualistic dispersers or antagonistic predators—as Jorge and Howe (2009) observed in scatter-hoarding rodents—and pre-dispersal seed predation can constitute a considerable bottleneck in regeneration (Sugiyama and Peterson 2013).

In summary, the effects of forest habitat degradation via anthropogenic disturbance and fragmentation are likely to cause changes in the regeneration ecology of tree populations, as well as population genetic changes that will influence their evolution.

1.4 This Thesis

1.4.1 The study site

Field work for this thesis was carried out on the Mambilla Plateau, an area of highland in the south-eastern corner of Nigeria that is connected to the Cameroon Highlands (FIGURE 1.3). While the altitude and soils of Mambilla suggest that it was once largely forested, anthropogenic pressure (overgrazing, fire, logging etc) has likely lead to a significant loss of old forest (Chapman and Chapman 2001, Adanu et al. 2010).

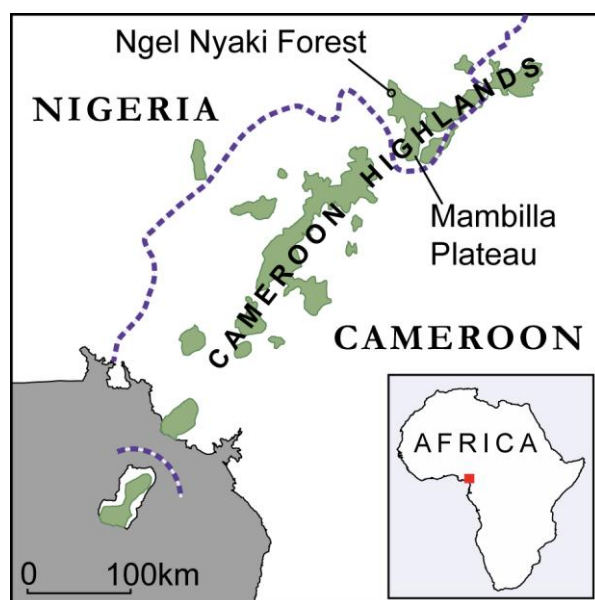


FIGURE 1.3 | A map of the Cameroon Highlands. Ngel Nyaki Forest—where most of this study’s research was conducted—is located in Nigeria. SOURCE: modified from Beck and Chapman (2008).

The bulk of the field work was carried out at the University of Canterbury’s field station at Ngel Nyaki Reserve, Taraba State, Nigeria. Ngel Nyaki is a moderately-large forest fragment, $\sim 7\text{km}^2$, situated on escarpment of the Mambilla Plateau (Chapman and Chapman 2001). Though some 15 significant forest fragments exist on the plateau, Ngel Nyaki is the most diverse in terms of both flora and fauna (Chapman and Chapman 2001, reviewed in Brookini et al. 2012). Briefly,

characterization of the vegetation at Ngel Nyaki by Chapman and Chapman (2001) unearthed a number of species new to Nigeria (*Isolona* cf. *deightonii* and *Ficus chlamydocarpa*) and to West Africa (*Pterygota mildbraedii*, *Anthonotha noldeae* and *Apodytes dimidiata*). Ngel Nyaki is also home to the rare chimpanzee subspecies *Pan troglodytes ellioti* (Beck and Chapman 2008, Oates et al. 2009) and of two, recently described, new frog species (Blackburn 2010, Blackburn et al. 2010).

The reserve is situated between N07°05' and E011°05' at an altitude of 1,400–1,600m above sea level. The mean annual rainfall of ~1800mm occurs mainly between mid-April and mid-October (Nigerian Montane Forest Project Rainfall data). Mean maximum/minimum monthly temperatures for the wet/dry season are 26.1/13.1°C, and 23.1/16.1°C, respectively (Upper Benue River Basin records). In addition to Ngel Nyaki Forest, sampling was also carried out the edge of the neighboring forest fragment Kurmin Danko (ca N07°06' E011°01') and close to the river known locally as Mayo Kamkam (ca. N07°07' E011° 04'), which are both situated in the general area close to Yelwa Village (FIGURE 1.4).

Ngel Nyaki was largely free of human disturbance until relatively recently (Chapman et al. 2004; pers. comm. Misa Zubairu). In the early 1900s (up until perhaps the 1940s), elephants were residents of Ngel Nyaki Forest. Leopards (*Panthera pardus*), lions (*Panthera leo*), and hyenas (Hyaenidae) also used to be present in the area, but these disappeared by the end of the 1970s as human presence increased. The forest also used to have large and conspicuous populations of primates and ungulates. Contemporary populations, however, are significantly reduced relative to their past sizes, though to species-specific degrees. Olive baboons (*Papio anubis*), Tantalus monkeys (*Chlorocebus tantalus*), and putty-nosed monkeys (*Cerocopithecus nictitans*) are still relatively common, but chimpanzees (*Pan troglodytes ellioti*) and larger ungulates are elusive and chiefly residents of the deep core in the forest (Chapman et al. 2004; pers. comm. Misa Zubairu).



FIGURE 1.4 | A map of the Mambilla Plateau in Nigeria's south-east. The location of Yelwa Village is indicated along with a number of key forests in the region. In this study, samples were sourced from the forest Ngel Nyaki and Kurmin Danko, as well as Akwaizantar. Trees were also sampled near the locally named river Mayo KamKam (close to Yelwa). **SOURCE:** modified from Chapman and Chapman (2001) with permission.

Deforestation of Ngel Nyaki has fortunately been very minimal. Slash and burn was attempted in prior decades (Chapman et al. 2004), but this has ceased in recent years. The main and continual threat to the forest is cattle: not only do cows enter into the forest—destroying the vegetation and eating seedlings—but the Fulani cattle herders annually burn the grassland surrounding the forest to encourage new growth—which likely limits the spread of seedlings into the grassland matrix and has the possibility of corroding the forest edge if burnings were to get out of control (Adanu et al. 2010).

1.4.2 The study species

Three tree species have been chosen to test the questions relevant to the conservation of Afromontane forests: These are *Lovoa trichilioides* (Harms.), *Entandrophragma angolense* (Welw.) and *Cordia millenii* (Bak.). *L. trichilioides* and *C. millenii* were chosen—amongst other reasons—because they are locally rare in Ngel Nyaki and appear to have a rather limited distribution in the forest. Because of this apparent rarity, I am confident that significant proportions—if not all—of the adult individuals in these two species have been sampled within their respective populations. The same cannot be said for *E. angolense*, whose distribution appears more extensive. However, these differences in spatial distribution lead me to study *E. angolense* as an interesting comparison to *L. trichilioides*, as the two are in the same family.

TABLE 1.1 | A summary of the focal tree species in this study. NOTES: ‘Distribution’ refers to the spatial arrangement of known adult trees in Ngel Nyaki (see text for detail); ‘Dispersal’ refers to the vector responsible for dispersing seed; ‘Shade tolerance’ refers to the ability of the species to be tolerant to shade; ‘Red List’ refers to the species conservation status as indicated by IUCN (see text).

Species	Family	Distribution	Dispersal	Shade tolerance	Red List
<i>Cordia millenii</i>	Boraginaceae	Clumped	Animal	Intolerant	Least concern
<i>Entandrophragma angolense</i>	Meliaceae	Wide spread	Wind	Tolerant	Vulnerable
<i>Lovoa trichilioides</i>	Meliaceae	Restricted	Wind	Tolerant	Vulnerable

All three tree species are under pressure from logging in Nigeria (Adekunle 2006, Adekunle et al. 2010) and *L. trichilioides* and *C. millenii* are important ethnobotanicals (Kayode 2005, 2006). Distributions of each species in Ngel Nyaki are illustrated in FIGURE 1.5. Additionally, *C. millenii* was sampled at two other locations close to Yelwa Village: Kurmin Danko Forest and the Mayo Kamkam River, and these locations are depicted in FIGURE 1.6. A brief summary of each tree species is given in TABLE 1.1.

Lovoa trichilioides is a member of the family Meliaceae, which are mahoganies; the wood of this tree is attractive and highly sought after for making furniture (Keay 1989). This desirability of *L. trichilioides* in the timber trade was another reason it was selected for this study; it is currently listed as Vulnerable on the IUCN Red List (IUCN 2013a). Though *L. trichilioides* has been reported to flower between the months of October–December, and fruit in March (Keay 1989), in Ngel Nyaki this species exhibits different phenological behavior (Nigerian Montane Forest Project data): the *L. trichilioides* of Ngel Nyaki may flower as late as February–May and fruit as late as September. Though there are a lack of direct tests on the pollination ecology of Meliaceae, the sweet and prominent scent of the flowers has lead to the suggestion that they are insect pollinated (Mabberley 2011); the breeding system of *L. trichilioides* is monoecious (Styles 1972). The fruits of *L. trichilioides* are small woody capsules ~2.5–5cm long with four segments that peel back to release the ballistic seeds; each seed has a single papery wing (Keay 1989). Trees can approach 45m in height (Keay 1989).

Entandrophragma angolense is also in the Meliaceae family, and thus a mahogany. Relative to other congeners, it has a wider distribution, widespread throughout African lowland rainforest, forest outliers and lower montane forests (Keay 1989). Adults can reach 48m in height (Keay 1989). An important timber tree, *E. angolense* is listed as Vulnerable on the IUCN Red List (IUCN 2013b). While flowering has been listed as occurring between November–March, and fruiting from January–May (Keay 1989), mature fruits have been recorded as early as October in Ngel Nyaki (Nigerian Montane Forest Project data). Like other Meliaceae, *E. angolense* is likely insect pollinated (Mabberley 2011, see above), and it also has a monoecious breeding system (Styles 1972). Fruits are woody 8–23cm long pods with four segments that peel back to release wind-dispersed seeds (like *L. trichilioides*, but *E. angolense* seeds are larger; Keay 1989).

Cordia millenii hails from the Boraginaceae family; it is a small tree that seldom reaches more than 12m high and is one of eight native species within this genus occurring in Nigeria (Keay 1989). Flowering occurs roughly around January–April, with fruit appearing in the months between April–November; fruits are ovoid with a pointed tip and an enlarged calyx forms a shallow cup at

their base (Keay 1989). While information on the breeding system of *C. millenii* appears non-existent, work on Neotropical *Cordia* by Opler *et al.* (1975) indicates that heterostyly and dioecism is common in this genera—though the degree of dioecy was found imperfect. These workers also noted that a large assemblage of insect pollinators (Hemiptera, Coleoptera, Diptera, Hymenoptera, Lepidoptera), but occasionally hummingbirds, visited Neotropical *Cordia*. Seed dispersal of *C. millenii* is largely dependent on primate dispersers—in particular, chimpanzees (Babweteera 2009). Babweteera (2009) demonstrated that if anthropogenic disturbance upsets primate assemblages, such that larger species are lost from local communities, there is a dramatic failure in seed dispersal of *C. millenii*. This was also correlated to a complete absence of juveniles in a disturbed versus a less disturbed forest. More importantly, Babweteera brought light to the fact that this species has potential to be easily driven to local extinction. This challenges the IUCN Red List status of *C. millenii* of Least Concern (IUCN 2013c); perhaps there *is* a need for concern given this species' ecological sensitivity and the threat from logging in Nigeria? Some authors do recognize a potential threat to *C. millenii* in Nigeria (Onyekwelu *et al.* 2008).

1.4.3 Thesis outline and research goals

The goal of this present thesis is to address the viability of populations of selected Afromontane tree species on Mambilla. The questions considered cover the genetic consequences of populations existing in a degraded and fragmented habitat, the issue of regenerating in the face of anthropogenic disturbance, and understanding the taxonomic significance of West African montane species. In CHAPTER 2, genetic diversity and potential structuring of populations is examined. Using conserved genetic primers, each of the three study species are thus compared to each other in an equivalent way. In CHAPTER 3, the taxonomic placement of Ngel Nyaki's *Cordia* species is assessed to determine its potential significance as a new taxon or evolutionary lineage. This is achieved using molecular markers sourced from both the nuclear and chloroplast genome. In CHAPTER 4, the possible variables impacting the regeneration of *Cordia* juveniles in Ngel Nyaki are addressed. These include seed dispersal, seed predation, and factors that may limit seedling survival. Finally, in CHAPTER 5, the findings of this present thesis are summarized and discussed in terms of conservation management in Ngel Nyaki Forest Reserve, but also their greater application to the conservation of Afromontane trees.

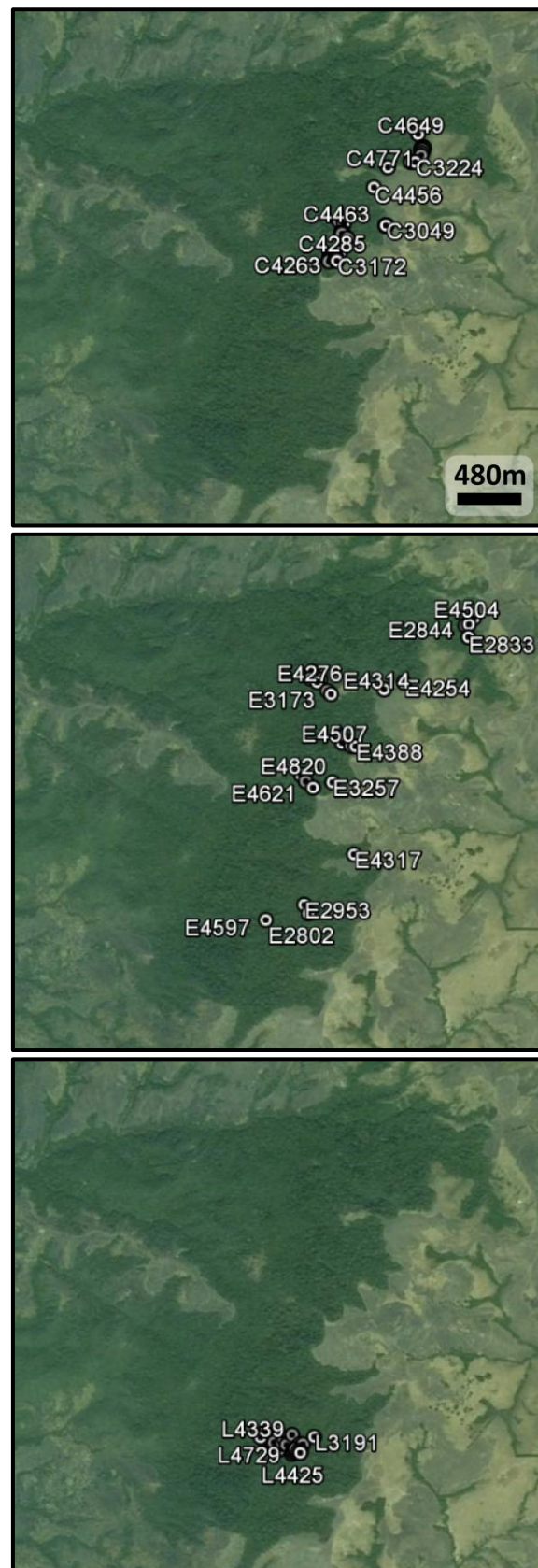


FIGURE 1.5 | Distribution of focal tree species in Ngel Nyaki. From top to bottom, known adults of *C. millenii* (C), *E. angolense* (E) and *L. trichilioides* (L) within Ngel Nyaki Forest. SOURCE: map from Google Earth (Google 2014).



FIGURE 1.6 | Distribution of *C. millenii* in the Yelwa area. Adults of *C. millenii* were found in three locations close to Yelwa Village (Mambilla Plateau). These were the forests of Ngel Nyaki and Kurmin Danko and the grassland near the river Mayo Kamkam. SOURCE: map from Google Earth (Google 2014).

CHAPTER 2

Genetic diversity patterns of tree populations in Ngel Nyaki Forest and on the Mambilla Plateau

Abstract

Fragmentation and anthropogenic disturbance threaten the future of forests across Africa by reducing the size of forested habitat and disrupting ecological communities. Decreases in population size are predicted to correspond to genetic diversity declines, which are expected to bode poorly for a population's evolutionary future. Yet the scientific literature regarding the genetic consequences of fragmentation in tree populations demonstrates that the ecological traits of trees can lead to very divergent responses amongst species. Further still, forests in montane Africa are expected to have a history of palaeoclimatic fragmentation, which complicates the effects of contemporary versus ancient fragmentation events on current population genetic processes. This study assessed genetic variation within and among three different montane tree species (*Cordia millenii*, *Entandrophragma angolense*, *Lovoa trichilioides*) found on the Mambilla Plateau, Nigeria. Conserved chloroplast SSR primers were used to provide directly comparable estimates of genetic variance of these three species that co-occur in Ngel Nyaki Forest. Additionally, wider sampling of *C. millenii* was conducted to assess how chloroplast variance might be dispersed between forests on Mambilla. Results demonstrated incredibly low diversity for all three species within Ngel Nyaki, and for *C. millenii* on Mambilla. This suggests that tree populations on the Mambilla Plateau may generally be highly depauperate of chloroplast diversity, which could be a product of historical bottlenecks caused by palaeoclimatic range shifts characteristic of Afromontane forest. Conservation efforts thus need to understand where diversity is partitioned across the landscape and how populations are connected via gene dispersal to mitigate potential impacts caused by anthropogenic disturbance.

2.1 Overview

2.1.1 *The montane forests of West Africa, forest fragmentation, and tree population genetics*

The loss and degradation of habitat is one of the biggest drivers of biodiversity loss globally (Kuussaari et al. 2009). While deforestation is a serious problem worldwide, loss of forest in Africa is particularly alarming because the amount of intact forest remaining is largely restricted to Central Africa (Norris et al. 2010). The most recent figures suggest that the annual loss of forest in Nigeria is 3.5% (Ajayi et al. 2008). As forests decline in area and become fragmented populations are undoubtedly put under stress. Smaller populations in stressful environments are likely to experience lower fitness and ill genetic effects due to inbreeding (see SECTION 1.3.2 for a review of genetic health in fragmented populations). Ergo, while a population of organisms may not go extinct at the onset of habitat degradation, genetic diversity may be sufficiently reduced to increase their chance of extinction in the future (Willi et al. 2006, Kuussaari et al. 2009).

As highlighted in SECTION 1.3.4, trees are unusual when compared to many other taxa because they vary in their responses to fragmentation. While many studies show forest degradation is driving undesirable genetic and fitness outcomes in tree populations (Boshier et al. 1995a, Ghazoul et al. 1998, Fuchs et al. 2003, Fernández-M and Sork 2007, Quesada et al. 2013), there are also cases where the observed effects were marginal or generally nonexistent (e.g. White et al. 2002, Lourmas et al. 2007, Born et al. 2008, Lobo et al. 2013, Noreen and Webb 2013), and cases where they are mixed (Jolivet et al. 2012). It is therefore unsurprising that Piotti (2009) described the literature as a “fifty-fifty trend” between expected and unexpected responses to fragmentation in tree populations. Also, the longer lifespans/generations of trees creates a delay in the onset of fragmentation consequences (reviewed in Aguilar et al. 2008 and Piotti 2009, but see Ashworth and Martí 2011).

Discrepancies in the results amongst tree population genetic studies lead Kramer et al. (2008) to propose the theory of the ‘Paradox of Forest Fragmentation Genetics’. This postulates that trees may break at least one of four key assumptions related to a population’s fitness in a fragmented landscape, which are: (a) fragment boundaries delimit population boundaries; (b) genetic declines manifest rapidly enough to be detected by researchers; (c) all tree species respond identically to fragmentation; and finally, (d) genetic declines supersede the ecological impacts of fragmentation on fitness. Therefore, it is also important to consider genetic data in conjunction with ecological factors (Kramer et al. 2008, Bacles and Jump 2011).

The processes that shape a tree population's genetic properties are diverse (Dick et al. 2008). Pollen flow and seed dispersal represent the movement of genes across the landscape, and if this movement is leptokurtic, will result in greater relatedness of seed sired or seedlings germinating near parental plants (Fleming and Heithaus 1981, Boshier et al. 1995a, Heuertz et al. 2003). The mating system of a population is highly important: i.e. are individuals outcrossing (receiving pollen from other individuals), or are they selfing (self-fertilizing their own seed)? Mating system impacts the genetic structure, the rate of inbreeding, and subsequently, inbreeding depression (Boshier et al. 1995b, Husband and Schemske 1996, Lacerda et al. 2008, Dick et al. 2008). Because seed dispersal is generally more restricted, limited seed dispersal could reduce the effect of long-distance pollen flow (Boshier et al. 1995a, Hardy et al. 2006).

The density of reproductive adults within a population can trigger different foraging behaviors in pollinators, which will determine the extent to which genes are moved across the landscape, outcrossing rates, and thus population genetic structure (Murawski and Hamrick 1991, Hardy et al. 2006, Ismail et al. 2012, Lobo et al. 2013). Structuring in the population can also be temporally and spatially variable across the landscape due to seasonal variance in flowering and the distribution of adults and genders (Boshier et al. 1995b, Dick et al. 2008). Reduced mate availability in small fragmented populations may lead to pollen limitation in self-incompatible/predominantly outcrossing plants, impacting fitness (Quesada et al. 2003, Glémin et al. 2008, Jones and Comita 2008). Inbreeding depression may also be particularly strong in trees during early life stages (Dick et al. 2008), and this can lead to erosion of the signature of inbreeding as selection weeds out less fit, inbred individuals (Fuchs and Hamrick 2010, Collevatti and Hay 2011).

However, the context of fragmentation in Ngel Nyaki and other forests on Mambilla is somewhat different to that of many other tropical forests. While the bulk of literature regarding forest fragmentation is very much focused on relatively recent anthropogenic disturbance creating isolated patches of forest (e.g. Aldrich and Hamrick 1998, Cosson et al. 1999, White et al. 2002, Quesada et al. 2003, 2004, Baranga 2004, Wieczkowski 2010, Ashworth and Martí 2011, Breed et al. 2012, Noreen and Webb 2013), forests on Mambilla have probably existed as fragments for centuries. Firstly, anthropogenic disturbance (in the way of logging) has been minimal in these forests (see SECTION 1.4.1), but most importantly, climatic processes over geological time have undoubtedly contributed to the patchy remnant patterns exhibited by forests on the Mambilla Plateau.

As discussed in SECTION 1.1, montane forests of Africa have exhibited fluctuations in size and distribution due to cycles of global warming and cooling (Hamilton and Taylor 1991, Maley 1996,

Elenga et al. 2000, Dupont et al. 2001). Eeley et al. (1999) demonstrated that montane forests in the KwaZulu-Natal Province, South Africa, became extremely fragmented during the last glacial maxima (~18,000 years B.P.), which was colder and drier than present. Afromontane forest distributions could therefore be seen as products of “ancient fragmentation” events due to paleoclimatic change. It therefore seems reasonable to assume that, given Ngel Nyaki has experienced little anthropogenic pressure until its more recent past (see SECTION 1.4.1), its current fragmentation status is largely a product of climatic and/or other natural processes.

2.1.2 Molecular markers in ecology

Investigating the genetic properties of tree populations requires the use of molecular tools to estimate the genetic variation within the populations. Different markers have their own set of strengths and weaknesses. While mitochondrial (mt)DNA is extremely useful in animal population genetic studies (given its uniparental inheritance, small size, high copy number, conserved gene order, rapid substitution rates, and availability of primers), it is undesirable for work in plants (Provan et al. 2001). This is because plant mitochondria have slow substitution rates and complex recombination patterns—and are thus somewhat intractable to population genetic analysis (Provan et al. 2001, Ballard and Whitlock 2004).

AFLPs (amplified fragment length polymorphisms) are used regularly in plant genetic studies (reviewed in Meudt and Clarke 2007; but see Jump et al. 2008, Derero et al. 2011, and Jaramillo et al. 2011, for examples). This method employs restriction enzymes to cut the DNA and compare different banding patterns produced by resulting DNA fragments amongst individuals (Meudt and Clarke 2007). AFLPs are attractive because they provide a means to easily obtain many loci with no need for *a priori* genetic information on the species of interest, are useful in polyploids (in which co-dominant data can be hard to score), are a relatively cheap process, and sample loci from across the genome (Meudt and Clarke 2007). However, some of the downsides to AFLPs are that they produce dominant data, homoplasy of similarly sized bands can underestimate genetic variance, and they can be severely impacted by contamination (Selkoe and Toonen 2006, Meudt and Clarke 2007).

An attractive alternative to AFLPs are SSRs (simple sequence repeats), also known as microsatellites. SSRs are reasonably short repetitive DNA sequences of 1–6 nucleotides (Selkoe and Toonen 2006). The genesis and evolution of SSRs is based on a dynamic interplay between the rate at which DNA polymerases ‘slip’ while replicating the repeat region (adding or deleting repeats) and the rate at which mismatch repair mechanisms correct these errors (Kalia et al. 2011).

Because SSRs are found in both non-coding and coding regions, and are found in the nuclear, mitochondrial, and chloroplast genomes, we classify SSRs based on their size, location, and type of repeat (Kalia et al. 2011).

The benefit of SSR markers are that they are easy to use, contain high information content if the locus is sufficiently polymorphic, are resistant to cross-species contamination, and provide co-dominant genotype data (Selkoe and Toonen 2006). However, there are challenges to working with SSRs, in that (while cross-amplification amongst species is possible) species-specific markers often require isolation due to limited taxonomic breadth of a given primer set, the mutational mechanism is not well understood, homoplasmy and allele dropout (i.e. alleles that have accrued a mutation in the primer binding region that do not amplify during PCR) may occur, and (unless we specifically want them to) we must ensure the loci are not under selection or in gametic disequilibrium (Selkoe and Toonen 2006).

2.1.3 Universal cpSSRs for comparative studies of tree populations

Due to the popularity of SSRs—specifically nuclear (n)SSRs—with molecular ecologists, there is an increased need to develop primer sets that are more universal to facilitate comparisons amongst multiple species in a direct way (Barbará et al. 2007). Comparative work by Barbará et al. (2007) demonstrated that nSSRs have limited transferability, in that even if primers can cross-amplify amongst different taxa—and across different taxonomic levels—it does not necessarily mean a researcher will be able to obtain useful polymorphisms at those loci for meaningful analysis due to ascertainment bias (i.e. the purposeful selection of highly polymorphic loci during primer development) in the original species. However, where possible, the use of cross-amplifiable primers allows for interesting interspecies comparisons of population genetic processes, as has been done in *Pitcairnia* (Palma-Silva et al. 2011) and *Alcantarea* (Barbará et al. 2008) bromeliad species, shorebirds (Küpper et al. 2008), domestic and wild cats in Europe (Lecis et al. 2006), Neotropical cats (Trigo et al. 2008), ants (Mäki-Petäys et al. 2005), pine trees (Clark et al. 2000), and *Erythrophleum* tree species in Central Africa (Duminil et al. 2010).

The issue of cross-amplification over greater taxonomic distances can be overcome using more conserved primer sets. While this may be hard for nuclear loci, the chloroplast of plants provides an excellent resource for molecular ecologists. In terms of its characteristics, the chloroplast genome can be described as analogous to the mitochondrial genome of animals: it has a conserved gene order, uniparental inheritance, and an absence of heteroplasmy and recombination by-and-large (Provan et al. 2001). While low substitution rates of chloroplasts—and thus lack of molecular

variance—have limited their use in population studies in the past, development of more rapidly evolving chloroplast (cp)SSRs provides another marker option for those wishing to study plant genetics (Provan et al. 2001).

cpSSRs can be used to quantify genetic diversity and population structuring, and because their effective population size is half that of the nuclear genome in hermaphroditic outcrossing plants, they can be used to identify historical bottlenecks, founder events, and drift (Provan et al. 2001). However, because of their uniparental inheritance (Provan et al. 2001), cpSSRs can only give us information about genetic processes in the sex that donates the chloroplast genome (and the same is true for mtDNA; reviewed in Whitlock and McCauley 1999). A real benefit of cpSSRs though is the fact that primers can be located in conserved flanking regions of the loci, which means that cross-amplification of primers may be considerably broader (see Weising and Gardner 1999).

2.2 Research goals

In this chapter, the issue raised by the Paradox of Forest Fragmentation Genetics is addressed: are the genetic responses of tree species to forest fragmentation similar? Samples collected from adult trees in all three study species (*Cordia millenii*, *Entandrophragma angolense*, *Lovoa trichilioides*) were sourced from Ngel Nyaki Forest (a moderately sized forest fragment), but also the nearby Kurmin Danko forest (also a forest fragment) and the more distant Mayo Kamkam locale. Specifically, the following questions were asked: (i) Is the genetic diversity in each respective tree population similar? (ii) How is this genetic diversity distributed within each respective population? To my knowledge, this is likely one of the few studies that addresses genetic variation in multiple co-occurring tropical tree species in a single forest locale (but see Stacy et al. 1996, Hardy et al. 2006, Duminil et al. 2010). Understanding which tree populations have more or less genetic diversity could help prioritize management in this disturbed forest. Furthermore, knowledge of how any genetic diversity is distributed across the landscape may help guide future management efforts: for example, if genetically similar trees are clumped together in space, it is worth considering artificially moving propagules during management efforts to more evenly disperse genetic variance.

2.3 Methods

2.3.1 Sample collection and lab work

For the purpose of this study, Ngel Nyaki, Kurmin Danko, and Mayo Kamkam are referred to as locales in the greater Yelwa region. Sampling took places at various times: (a) April 2012, (b) September 2012, (c) June–September 2013, and (d) December 2013; (a), (b), and (d) sampling efforts were conducted by field assistants and (c) personally by me. Individuals for each species (in Ngel Nyaki) were located along, or close to, phenological transects established by the Nigerian Montane Forest Project (pers. comm. Hazel M. Chapman). Individuals for *C. millenii* in the greater Yelwa region were located with the help of field assistants. A specific identification number was given to each tree; this was a small metal tag that was nailed into the trunk. Where possible, leaf tissue was collected; if it was not possible to collect a tree's leaves, a cambium core was taken following a protocol analogous to that employed by Colpaert et al. (2005). Briefly, a leather punch was used to remove a cylindrical core from the trunk. The cambium was then sliced very thinly into discs and stored with silica gel to remove moisture. The outer layer of cambium and the bark was replaced into the hole to prevent infection. As soon as possible, samples were frozen at -20°C for long-term storage.

Additionally, an individual of *C. millenii* was obtained from Akwaizantar Forest (~43km south of Ngel Nyaki, $\sim\text{N}06^{\circ} 52' \text{ E}10^{\circ} 55'$; see FIGURE 1.4) by means of a herbarium specimen sourced from Kew Royal Botanical Gardens. The addition of this individual, MIL31795, into the data set provides a perspective of between forest variation on the Mambilla Plateau for *C. millenii* populations. Though it would have been ideal to have sampled more forests, and also have representatives of *E. angolense* and *L. trichilioides* from outside Ngel Nyaki, this was not practical.

Various DNA extraction methods were assessed because acquiring useable DNA from wood tissue is challenging (Colpaert et al. 2005, Zytynska et al. 2011). These included variations on the CTAB method (e.g. see Brunner et al. 2001) as well as the use of commercial kits. All extractions began with the pulverization of ~5–6mg of leaf or cambial tissue—using metal beads and a Retsch Mixer Mill MM400—into a fine powder. In the end the best workable extractions were obtained using either the PowerPlant® DNA Isolation Kit (MoBio Laboratories) and a personal variation on a modified CTAB method described in Brunner et al. (2001; described below). The PowerPlant® extractions were carried out as per manufacturer instructions, using the troubleshooting tips as required.

The bulk of CTAB extractions were based on the protocol used by Brunner et al. (2001) and performed as follows: 500mg/L spermidine and 5% PVPP were added to an autoclaved extraction

buffer consisting of 100mM Tris-HCl (pH 8), 25mM EDTA, 2M NaCl, 2% CTAB, and 2% PVP. To each tube containing ground tree tissue, 650µL of this extraction buffer was added, along with 2% β-mercaptoethanol and 3.5µL 10mg/mL ProtK. The samples were then incubated at 65°C for 30 minutes and then spun for 10 minutes at 13,000rpm. The supernatant was collected, transferred to a new tube, combined with 200µL MilliQ water and 8µL 5mg/mL RNase, and then left to incubate at 37°C for 10 minutes. Additional 200µL MilliQ water was added at this step because the supernatant volume was small and a greater volume was required to prevent loss of the extraction in downstream steps. To this, 700µL of 25:24:1 phenol-chloroform-isoamyl alcohol solution was added. Tubes were inverted several times and spun for 8 minutes at 13,000rpm. The supernatant was transferred to a new tube. A final 700µL phenol separation was carried out (as above) and the supernatant transferred to another clean tube, to which 1.5 volumes of cold isopropanol (usually ~600µL) was added. The samples were left at -20°C for at least 1 hour (but sometimes over night), after which they were spun at 13,000rpm for 10 minutes. The DNA pellet was washed twice with ice-cold ethanol and the tubes put briefly onto a hot plate set at 65°C to rapidly dry off this ethanol. The DNA was then dissolved in 80µL warmed TE buffer and incubated at 65°C for 5 minutes to help dissolve DNA and denature any residual DNases. The quantity and quality of DNA extract were tested using a NanoDrop™ spectrophotometer. In general, DNA yields from both the PowerPlant® and CTAB method yielded ~20–40ng of DNA. Absorbance readings at 230nm and 280nm varied widely between samples, and it was evident that many of the extractions contained organic compound contamination (indicated by high 230/260 ratios).

Initially, *Taq* polymerase from Bioline was used in the PCR. However, it proved difficult to get consistent amplification from the samples. The samples were therefore diluted (e.g. to 0.1 and 0.01× initial concentration), to try and attenuate the effect of any inhibitory compounds present, but with little success. After much trial and error, the KAPA3G Plant PCR Kit manufactured by Kapa Biosystems was used. The DNA polymerases in this PCR kit have been specifically engineered for improved tolerance to inhibitors common in plant extracts—e.g. polyphenolics and polysaccharides. While Kapa Biosystems recommends a reaction volume of 50µL for difficult samples most of the samples worked at a reaction volume of 20µL using the reagent mix: 10µL KAPA Plant PCR Buffer (2×), 0.3µM each primer, 5mM additional MgCl₂, ~20ng DNA, 0.2µL KAPA3G Plant DNA Polymerase, and PCR water as required, per reaction. Some reactions required the addition of KAPA3G Enhancer solution at final concentration of 1×.

The primers implemented in this study were sourced from a conserved set of chloroplast SSR primers for dicotyledonous angiosperms reported in Weising and Gardner (1999). These cpDNA SSRs are named the “ccmp” (consensus chloroplast microsatellite primers) loci and are situated

within intronic/intergenic regions, or just 3'/5' to, various chloroplast genes. After screening all the loci it was determined that only ccmp2, ccmp3, ccmp4, ccmp5, ccmp6, ccmp7, and ccmp10 would amplify in my study species—providing a total of seven loci. The PCR conditions were as recommended by Kapa Biosystems, but it was found a longer extension phase provided more reliable amplification: 3 minutes at 95°C (initial denaturing); 40 cycles of 20 seconds at 95°C (denaturing), 15 seconds at 50°C (annealing), and 45 seconds at 72°C (extension); completed with 30 seconds at 72°C (final extension). For some particularly difficult samples it was found that increasing the annealing time to 20 seconds, and the extension time to 50 seconds, assisted successful amplification. Size differences amongst loci were initially examined visually by running samples on a 1% agarose gel. All loci fell within ~100–250bp long, but there were evident size disparities amongst loci and between species. To allow pooling of labeled products for genotyping, primers that had sufficient size differences were paired to be marked with the same coloured fluorescent tag. Information regarding these selected primers (e.g. their position in the chloroplast genome and assigned coloured tags) is listed in TABLE 2.1.

Genotyping was carried out using an ABI Prism 3130xl Genetic Analyzer. PCR products were run on a 1% agarose gel to approximate their concentration. Extremely bright bands were diluted to a final concentration of 0.1×, whilst weaker bands were not. To MultiMax™ 96 well plates, 0.6µL of PCR product for a given locus was added for genotyping; in some exceptions 1µL of PCR product was used for samples showing extremely weak bands. After a preliminary run—in which each locus was genotyped separately for a small subset of individuals—it was determined loci could be pooled based on their size differences.

For all species, loci could be pooled into two groups: (a) loci ccmp2, ccmp3, ccmp4, and ccmp5; and (b) loci ccmp6, ccmp7, and ccmp10. Into each well containing PCR product, 12uL HiDi™ (Applied Biosystems) and 0.3µL 500 LIZ™ (Gene Scan™) was added. Reads were analyzed in the program Gene Marker v1.97 (SoftGenetics, LLC, CA, USA). As in the original work by Weising and Gardner (1999), alleles for each ccmp locus were scored as the second to largest peak. Because the genotypes produced by cpSSRs are haploid, there was no need to assess for null alleles as should be done for nuSSRs (Selkoe and Toonen 2006).

2.3.2 Genetic analyses

The effective number of haplotypes (n_e) and the haplotypic diversity (H_E) were calculated for each population of tree species as follows:

$$n_e = \frac{1}{\sum p_i^2} \quad \text{EQUATION 2.1}$$

$$H_E = \frac{n}{n-1} \times \left(1 - \sum p_i^2\right) \quad \text{EQUATION 2.2}$$

Where n is the number of individuals assessed per species' population, and p_i is the frequency of allele i in a population.

Average genetic distances amongst individuals within each tree species' respective population were measured using the metric \bar{D}_{SH}^2 that is described in Vendramin et al. (1998), elaborated from Echt et al. (1998). It is based on a step-wise mutational model (Goldstein et al. 1995) and measures the squared pair-wise haplotype differences amongst all possible pairs of individuals using absolute size differences in their alleles:

$$\bar{D}_{SH}^2 = \frac{1}{[n(n-1)]/2} \times \frac{1}{L} \times \sum_{i=1}^n \sum_{j=i+1}^n \left[\sum_{k=1}^L |a_{ik} - a_{jk}| \right]^2 \quad \text{EQUATION 2.3}$$

Where n is the number of individuals; L is the number of loci; a_{ik} and a_{jk} are the allele sizes for individuals i and j at locus k . This method requires a comparison of allele size differences for every possible pair-wise combination of individuals in the population. First, for a given pair of individuals, absolute size differences are calculated for each locus. These differences are summed and the total squared. This process is repeated for each possible pair of individuals to obtain a value representing the total amount of distance between individuals in the sample. This is then averaged across pairs of individuals, $[n(n-1)]/2$ total number of unique pairs, and standardized for the number of loci, $1/L$. The \bar{D}_{SH}^2 statistic provides an easy to interpret measure of genetic dissimilarity amongst individuals at cpSSR loci, as it simply reflects the average number of bases that are different between individuals within a population.

TABLE 2.1 | Details of SSR (ccmp) loci. Location, repeat motif, and primer sequences for each locus within the chloroplast genome was sourced from Weising and Gardner (1999). Choice of coloured tag for each primer was based on differences in product size (as observed from gel electrophoresis).

¹ N.B. The repeat motif mentioned here are those deduced from the source species of these primer sets, i.e. tobacco, and are not necessarily the same in *C. millenii*, *E. angolense*, or *L. trichilioides*.

Primer	Location	Repeat motif ¹	Primer sequence	Dye/Colour	T _a
ccmp2	5' to <i>trnS</i>	(A) ₁₁	5'-GATCCCGGACGTAATCCTG-3' 5'-ATCGTACCGAGGGTTTCAAT-3'	6-FAM/Blue	50°C
ccmp3	<i>trnG</i> intron	(T) ₁₁	5'-CAGACCAAAAGCTGACATAG-3' 5'-GTTTCATTTCGGCTCCTTTAT-3'	PET/Red	50°C
ccmp4	<i>atpF</i> intron	(T) ₁₃	5'-AATGCTGAATCGAYGACCTA-3' 5'-CCAAAATATTBGGAGGACTCT-3'	6-FAM/Blue	50°C
ccmp5	3' to <i>rps2</i>	(C) ₇ (T) (T) ₅ C(A) ₁₁	5'-TGTTCCAATATCTTCTTGTCATTT-3' 5'-AGGTTCCATCGGAACAATTAT-3'	VIC/Green	50°C
ccmp6	ORF77-ORF82 intergenic	(T) ₅ C(A) ₁₇	5'-CGATGCATATGTAGAAAGCC-3' 5'-CATTACGTGCGAACTATCTCC-3'	NED/Black	50°C
ccmp7	<i>atpB-rbcL</i> intergenic	(A) ₁₃	5'-CAACATATACCACTGTCAAG-3' 5'-ACATCATTATTGTATACTCTTTC-3'	PET/Red	50°C
ccmp10	<i>rpl2-rps19</i> intergenic	(T) ₁₄	5'-TTTTTTTTTAGTGAACGTGTCA-3' 5'-TTCGTCGDCGTAGAAATAG-3'	VIC/Green	50°C

2.4 Results

The results from the ccmp SSR analysis suggest a genetically depauperate chloroplast gene pool in the three tree species under question. Of the seven loci examined, none of these were polymorphic in *C. millenii* or *E. angolense*, and *L. trichilioides* was only polymorphic at ccmp6. Allele sizes for each locus in each of the species are recorded in TABLE 2.2, and haplotype frequencies are detailed in TABLE 2.3. Given that all loci were monomorphic in *C. millenii* and *E. angolense*, each population only had a single haplotype (C1 and E1, respectively). In contrast, *L. trichilioides* was polymorphic at ccmp6, but only for two alleles: 120bp (frequency of 0.08) and 121bp (frequency of 0.92). Therefore, respective haplotype frequencies are identical to the allele frequencies at ccmp6 (L1, 0.08; L2, 0.92). In general, each ccmp locus exhibited a similar size range across all species. However, ccmp6 in *C. millenii* was dramatically smaller than *E. angolense* and *L. trichilioides* (70 versus 124 and 120/121bp, respectively), and reasonably smaller at ccmp2 (185 versus 213 and 206bp, respectively). Alleles of *E. angolense* and *L. trichilioides* were also more similar in size range to each other than compared to *C. millenii* at the other five ccmp loci, which is likely due to the former two being in the same family (Meliaceae) and thus more closely related.

The lack of genetic diversity at the ccmp cpSSRs in this study is reflected in the three genetic diversity indices used (TABLE 2.4). *C. millenii* and *E. angolense* both had values of $n_e = 1.00$, $H_E = 0.00$ and $\bar{D}_{SH}^2 = 0.00$. This is not surprising given there was no genetic variance detected in these populations. *L. trichilioides* exhibited slightly different values due to the small amount of variance present at ccmp6. In this population: $n_e = 1.17$, $H_E = 0.15$, and $\bar{D}_{SH}^2 = 0.02$, which are very small and suggest near to no variation.

Consideration of where individuals with the rarer L1 haplotype (Lov2924 and Lov4206) are in physical space shows that Lov2924 is the nearest neighbor of Lov4206 (which is somewhat isolated with respect to the other trees) and the two are separated by 66m. Contrastingly, the nearest neighbor of Lov2924 is not Lov4206, but rather Lov3107, 33m away. The DBH (diameter at breast height, 150cm high) of both trees suggests that Lov2924 is reasonably younger than Lov4206 (0.64m versus 1.07m), and may possibly suggest that the former is the progeny of the latter. However, this cannot be said with certainty, and a thorough test of parentage would need to be conducted to confirm this.

TABLE 2.2 | Allele sizes for ccmp loci, and their frequencies, in focal tree species.

Alleles ranged from 70–213bp, depending on the locus and the species. The vast majority of loci exhibited fixation; frequency in the sampled populations are represented in “()”. ^A Indicates *C. millenii* sourced solely from Ngel Nyaki; ^B indicates *C. millenii* pooled across Ngel Nyaki, Mayo Kamkam and Kurmin Danko, i.e. the collective region around Yelwa Village; ^C indicates *C. millenii* from Akwaizantar. Sample sizes denoted by *n*.

Locus	<i>C. millenii</i> ^A	<i>C. millenii</i> ^B	<i>C. millenii</i> ^C	<i>E. angolense</i>	<i>L. trichilioides</i>
Ccmp2	185 (1.00)	185 (1.00)	185 (1.00)	213 (1.00)	206 (1.00)
Ccmp3	106 (1.00)	106 (1.00)	106 (1.00)	104 (1.00)	105 (1.00)
Ccmp4	127 (1.00)	127 (1.00)	127 (1.00)	120 (1.00)	120 (1.00)
Ccmp5	102 (1.00)	102 (1.00)	102 (1.00)	92 (1.00)	90 (1.00)
Ccmp6	70 (1.00)	70 (1.00)	70 (1.00)	124 (1.00)	120 (0.08) 121 (0.92)
Ccmp7	125 (1.00)	125 (1.00)	125 (1.00)	136 (1.00)	137 (1.00)
Ccmp10	109 (1.00)	109 (1.00)	109 (1.00)	107 (1.00)	107 (1.00)
<i>n</i>	37	42	1	25	26

TABLE 2.3 | Frequency of haplotypes for ccmp loci. Because all ccmp loci were monomorphic in *C. millenii* and *E. angolense*, their populations only had a single haplotype. Comparatively, two haplotypes existed in *L. angolense*. Haplotypes are written in the numeric order of ccmp loci, that is: ccmp2, ccmp3, ccmp4, ccmp5, ccmp6, ccmp7, ccmp10. Frequency and sample size (*n*) for *C. millenii* sampled from within Ngel Nyaki only, the Yelwa Region, and Akwaizatar (respectively, c.f. TABLE 2.2) are separated with a “/”.

Species	Haplotype	Allelic composition	Frequency	<i>n</i>
<i>C. millenii</i>	C1	185/106/127/102/70/125/109	1.00/1.00/1.00	37/42/1
<i>E. angolense</i>	E1	213/104/120/92/124/136/107	1.00	26
<i>L. trichilioides</i>	L1	206/105/120/90/120/137/107	0.08	25
	L2	206/105/120/90/121/137/107	0.92	

TABLE 2.4 | Genetic diversity measures for ccmp data. The effective haplotype number (n_e), haplotype diversity (H_E), and the within population average genetic distance between individuals (\bar{D}_{SH}^2) are detailed below for all three study species in Ngel Nyaki (“NN”). Measures for *C. millenii* were also calculated for the greater sampling area in the greater Yelwa Region (“Yelwa”, c.f. TABLE 2.2). The sample size is denoted by n .

Species	n_e	H_E	\bar{D}_{SH}^2	n	Locale
<i>C. millenii</i>	1.00	0.00	0	37	NN
<i>C. millenii</i>	1.00	0.00	0	42	Yelwa
<i>E. angolense</i>	1.00	0.00	0	25	NN
<i>L. trichilioides</i>	1.17	0.15	0.02	27	NN

2.5 Discussion

The primary objective of this study was to assess levels and distribution of genetic diversity in three tree species in the montane forest of Ngel Nyaki, but also across forests on the Mambilla Plateau. The conserved ccmp primers were chosen to provide a directly comparable measure of genetic properties between populations of species due to the conserved nature of the primer binding sequences. This is of benefit to molecular ecological studies because many nuclear SSR primer sets have limited transferability (Barbará et al. 2007). In addressing the Paradox of Forest Fragmentation (Kramer et al. 2008), such point-blank contrasting allows testing of whether the population genetic variances of different tree species, in a single disturbed and fragmented forest, exhibit similar or disparate responses.

Results obtained by amplification of seven cpSSR loci suggest that the Ngel Nyaki populations of *C. millenii*, *E. angolense* and *L. trichilioides* are depauperate of genetic variation in their chloroplast genomes; the same is true for *C. millenii* external to Ngel Nyaki (TABLES 2.2–2.4). The former two species are the worst off—showing complete fixation at all loci in question—whilst *L. trichilioides* does exhibit variation at the ccmp6 locus. Still, this variance at the *L. trichilioides* ccmp6 is minute, and the low \bar{D}_{SH}^2 implies the average difference in haplotype amongst individuals is near zero bases. Because the selected ccmp loci exhibited minimal amounts of genetic variance, measures of genetic structure across the forest and amongst forests on the Mambilla Plateau could not be assessed.

The lack of genetic variance observed in all three focal species is both surprising, but at the same time not unreasonably improbable. Tree populations can harbor significant amounts of genetic variance in their chloroplast genomes. In six populations of Moroccan *Cedrus atlantica* high levels of within population diversity was observed using six cpSSRs: mean n_e was found to be 12.40, mean H_E was 0.95, and the \bar{D}_{SH}^2 was 11.46 (Terrab et al. 2006). A study on *Pinus pinaster* found high mean levels of n_e (3.750) amongst 10 populations, but with a more moderate mean H_E (0.737) and smaller \bar{D}_{SH}^2 (0.512) amongst individuals within populations (Vendramin et al. 1998). In another study of a pine species (*Pinus resinosa*), using seven cpSSR loci, Echt et al. (1998) observed one population that had incredibly high haplotype richness ($n_e = 8.397$, $H_E = 0.920$), further illustrating the cpSSRs can harbor considerable amounts of observable genetic diversity.

Studies that have specifically used ccmp loci to assess population genetic properties have found them to be useful diagnostic markers for assessing variance and population subdivision across plant taxa. Quintela-Sabarís et al. (2011), in a study on the phylogeographic patterns of the shrub *Cistus ladanifer* (Cistaceae), found that up to four distinct haplotypes per populations could be found using just two loci (ccmp2 and ccmp3). Using ccmp2, ccmp3, ccmp4, ccmp5, ccmp10 (and one other cpSSR), Cubas et al. (2005) demonstrated that populations of *Ulex* species. (Fabaceae) could be quite rich in haplotypes (i.e. many had H_E values >0.50). Rendell and Ennos (2003) found within population variance at ccmp4 and ccmp10 in *Ilex aquifolium* (Aquifoliaceae). Intrapopulation diversity for ccmp2 alone was also observed in the Afrotropical tree *Milicia excelsa* (Dainou et al. 2010). This begs the question as to why no (or close to zero) variation was found in the three focal species of this study, in which sample size per population and number of loci assessed was comparable—if not greater—than many of the studies listed above.

One possible explanation could be due to the sampling effort of this study, in terms of both genetic sampling and the scale at which sampling was conducted. The studies cited above tested a number of primer pairs (2–24, depending on the study) for their respective study species and populations, but it was common (though not in all cases) for a number of these loci to be found monomorphic, or exhibit inconsistent amplification, following initial screenings (Echt et al. 1998, Vendramin et al. 1998, Clark et al. 2000, Rendell and Ennos 2003, Cubas et al. 2005, Terrab et al. 2006, Duminil et al. 2010, Quintela-Sabarís et al. 2011). While universal cpSSRs provide an excellent resource to molecular ecologists, in that no effort has to be made to design primer sets for study species (Provan et al. 2001), it does come with the potential caveat of lacking polymorphisms. This is due to ascertainment bias: that is, primer sets designed to identify variation in a particular target species fail to identify meaningful polymorphisms when cross-amplified in a non-target species (Selkoe and Toonen 2006, Barbará et al. 2007).

The ccmp loci that were implemented in this study were developed initially in *Nicotiana tabacum* (tobacco), for general use in dicotyledonous angiosperms by Weising and Gardner (1999). However, even within their own paper, the authors illustrated that the level of variation detected with these primers differed amongst species, genera, and higher taxonomic levels. Consequently, it may be that by pure chance none of the loci selected for assessing the genetic diversity of *C. millenii*, *E. angolense* and *L. trichilioides* in Ngel Nyaki Forest are actually variable, and it does not rule out the possibility that genetic variance exists within each species' chloroplast genome. Thus, the paucity of variability could be due to inadequate genetic sampling of the populations. Furthermore, ccmp loci in the ash *Fraxinus excelsior* were suggested to exhibit low rates of mutation (Heuertz et al. 2004), which may hinder ability to detect genetic variants.

The other possibility is that the scale at which sampling took place was inadequate to capture allelic variants at the chosen ccmp loci in these species. Lira et al. (2003) examined seven populations of the rare tropical tree *Caesalpinia echinata* across its range in Brazil. They implemented a combination of ccmp and CECF (a species-specific set of) cpSSR primers to obtain haplotype data from the fragmented populations. It was discovered that in all but two of the seven populations, populations were fixed for a single haplotype, and those with multiple haplotypes did not share them with any other population. As a consequence, most populations had an H_E value of zero, whilst the two polymorphic populations had values of 0.385 and 0.209. This illustrates a scenario where diversity is predominantly between the populations as opposed to within them. Another example is the range-wide study of genetic variance in *Abies* pine species by Clark et al. (2000): despite sampling fewer than 10 individuals per population, by sampling across many populations, these authors were able to observe high levels of haplotype and gene diversity in just two cpSSR loci.

One limitation of this present study was the lack of sampling external to Ngel Nyaki Forest for *E. angolense* and *L. trichilioides*. While individuals of *C. millenii* were found at Mayo Kamkam and Kurmin Danko, it is possible that these locales are geographically too proximate to Ngel Nyaki to actually be counted as separate populations. Thus, little to no haplotypic diversity was detected, but need not mean that other trees of the same species on the Mambilla Plateau will be genetically identical to those in Ngel Nyaki at the ccmp SSRs. Nevertheless, it was surprising that the single *C. millenii* sampled from Akwaizantar (~43km away from Ngel Nyaki) had the exact same haplotype as those in Ngel Nyaki. This observation adds weight to the argument that ccmp loci may not be a reliable indicator of chloroplast genetic variance in the three study species. However, one would probably expect to see at least some differentiation between populations (Clark et al. 2000, Lira et al. 2003)

There does, however, exist a biological explanation as to why the three species in this study all exhibit similar genetic patterns in their populations, and that populations from different locales share the same haplotypes. As discussed in SECTION 1.1, the montane vegetation of Africa has experienced cyclic history of expansion and contraction over the last 800,000 years that correlate to patterns of glaciations (Maley 1996). Contraction of vegetation during interglacial periods would lead to shrinkage in population size and disconnection between them. Because population genetic theory posits that small, isolated populations are expected to undergo severe drift and become randomly fixed for different alleles (Willi et al. 2006, Frankham et al. 2011), it seems logical that over evolutionary time small forest populations like those on the Mambilla Plateau are likely to undergo major loss of genetic variance—which is likely exacerbated by anthropogenic disturbance that degrades forests and surrounding habitat.

A pattern analogous to that described here was observed in common ash (*Fraxinus excelsior*) in Europe, in which it is believed glacial refugium populations contained low effective sizes and few genetic variants, leading to abrupt contemporary spatial genetic patterns in contemporary populations. Work in the Afrotropical *Milicia excelsa* has also demonstrated the role of glacial refuges in regional structuring genetic variation in contemporary populations in West and Central Africa (Dainou et al. 2010). Additionally, more restricted seed dispersal relative to pollen flow can cause greater structuring in chloroplast markers relative to nuclear markers, intensifying the effect of drift on chloroplast gene frequencies (Levy and Neal 1999, Petit et al. 2005). Drift will also be higher for chloroplast genes because maternally inherited genomes have smaller effective populations than nuclear genomes (Provan et al. 2001, Charlesworth 2009).

The Paradox of Forest Fragmentation theory suggests that tree populations, contained in a forest fragment, need not suffer from genetic degradation because ecological factors may buffer the erosion of alleles and lead to variable species-specific genetic responses (Kramer et al. 2008). Furthermore, delayed sexual maturity and longer lifespan of trees vis-à-vis other plant species can help delay the onset of genetic consequences caused by fragmentation (Piotti 2009, Ashworth and Martí 2011). Given that the last glacial period was ~12,000–90,000 years B.P. (Hamilton and Taylor 1991), there has undoubtedly been a considerable number of generations that have passed since montane forests have likely been retreating to greater altitudes. Isolation of Ngel Nyaki is likely due to climatic retreat, as opposed to human-mediated destruction of forest or disturbance (see SECTION 1.4.1), and as such, this forest represents an ancient fragmentation event. All species have seeds that are unlikely to be dispersed considerable distances between forests. The seeds of *E. angolense* and *L. trichilioides* are both wind dispersed (Keay 1989), and the seeds of *C. millenii* are fairly large (Keay 1989, Dutton et al. 2014) and unlikely to be dispersed considerable distances

given the low probability of long-distance seed dispersal for larger seeds (Janzen 1988, Wunderle 1997, Hardy et al. 2006). Because of this, chloroplast lineages may have been confined within Ngel Nyaki for considerable time, gone through genetic bottlenecks and experienced drift, resulting in all three focal species exhibiting a similar genetic trend.

In regard to the lack of diversity observed in *C. millenii* adult trees found outside Ngel Nyaki (that is, at Mayo Kamkam, Kurmin Danko, and Akwaizantar), the most likely explanation—especially given the relatively close proximity of Mayo Kamkam and Kurmin Danko to Ngel Nyaki—is that in the past these locations were once all interconnected via intervening forest habitat. The ancestors of the present day *C. millenii* found within the study region likely underwent a genetic bottleneck before these locales became isolated. Consequently, the contemporary cpSSR haplotypes in the adult *C. millenii* found in all three locales are identical, despite the physical disconnection of forests they occur in. This is in accord with the proposition that cpDNA haplotypes of Afrotropical trees may be regionally structured based on ice-age retreat (Dainou et al. 2010).

Genetic comparisons of multiple co-occurring tropical tree species within the same community are not common in the literature. One example is the work by Hardy et al. (2006) in their comparisons of genetic data sets from 10 Neotropical tree species that shared the same forest fragments. In their analysis, they used the S_p statistic, a measure of the spatial genetic structure (SGS), which can be used as a comparative metric amongst different populations. They demonstrated that SGS of different species was related to propagule mass, suggesting that the process of seed dispersal may limit the flow of genes across the landscape. However, these workers relied on a range of different markers (allozymes, nSSRs, RAPDs, and AFLPs) to conduct their analysis. While some species were tested with multiple markers, others were only tested with one type. The data demonstrated that historical gene flow could be estimated reasonably well with nSSRs, RAPDs and AFLPs, but allozymes were poor at doing so. However, given the different characteristics of the marker types, and given that different markers within a species sometimes produced contradictory conclusions (Hardy et al. 2006), it would be prudent that studies focusing on species comparisons strive to compare them with equivalent marker types and primer sets. Some examples where this had been done in plants include: *Pitcairnia* (Palma-Silva et al. 2011) and *Alcantarea* (Barbará et al. 2008) bromeliad species; species of *Abies* pine trees (Clark et al. 2000); Neotropical trees (Stacy et al. 1996); and *Erythrophleum* tree species in Central Africa (Duminil et al. 2010).

Gene flow in plant populations is achieved through the processes of pollen flow and seed dispersal (Crawford 1984). Both pollen flow and seed dispersal are leptokurtic, in that movement of

propagules some x distance away from a parent becomes less probable with increasing distance (Boshier et al. 1995a, Heuertz et al. 2003). Pollen flow, however, can be extensive (Dick et al. 2003, Lourmas et al. 2007, Ismail et al. 2012, Lobo et al. 2013) and the leptokurtic distribution may be spread over a greater distance relative to seed dispersal (Boshier et al. 1995a). Yet even when pollen flow is extensive, if seed fails to disperse far from the maternal tree, populations will inevitably become structured to some degree due to a correlation between maternally inherited genes and seed movement (Heuertz et al. 2003, Hardy et al. 2006).

Ultimately, the movement of genetic material across the landscape will be determined by a number of factors (reviewed in Dick et al. 2008) that include outcrossing rates and reliability and effectiveness of pollen and seed dispersal vectors (Boshier et al. 1995a, 1995b, Dick et al. 2003, Born et al. 2008), the phenological patterns of flowering in tree populations (Fuchs et al. 2003, Lobo et al. 2013), the physical distribution of individuals and genders (Murawski and Hamrick 1991, Stacy et al. 1996, Dick et al. 2003, Fuchs et al. 2003, Lacerda et al. 2008, Ismail et al. 2012, Quesada et al. 2013), and the extent to which inbreeding depression weeds out inbred seeds and juveniles (Jones and Hubbell 2006, Collevatti and Hay 2011, Fuchs and Hamrick 2011). Plant populations that lose genetic diversity will likely have increased extinction risk, especially if they are self-incompatible (Glémin et al. 2008, Jones and Comita 2008); however, in trees this may be buffered by their longer generation times (Petit and Hampe 2006).

Furthermore, if harmful alleles are not lethal and only slightly deleterious they may be able to escape the intensive selective filter experienced by juveniles and be passed into the next generation (Husband and Schemske 1996). Fragmentation can thus have serious implications on plant population fitness as the degradation of habitat will impact movement of dispersal vectors, availability of mates, and create edge effects (Fischer and Lindenmayer 2007, Aguilar et al. 2008, McConkey et al. 2012). In due course, three broad evolutionary responses may arise from fragmentation of plant populations: (i) populations will lose genetic diversity via drift; (ii) ecological change may increase the rate of adaptation in the fragmented landscape; or (iii) populations incur short-term adaptive changes that are evolutionarily maladaptive, leading to the future suicide of the fragment populations (Jacquemyn et al. 2012).

To summarize, this present work focused on exploring the differences in genetic diversity in three different tree species—*C. millenii*, *E. angolense*, and *L. trichilioides*—in fragmented montane forests in Nigeria. A set of universal angiosperm cpSSR primers was employed for this purpose to provide comparable insights amongst the different populations that co-occur within the focal forest Ngel Nyaki, and to investigate potential variance in *C. millenii* in locales outside Ngel Nyaki.

Results indicate that populations of *C. millenii* and *E. angolense* are completely depauperate of genetic variance at the seven loci in question, whilst *L. trichilioides* exhibited a small amount of variance that contributed diminutively to the population's overall diversity.

Many studies regarding habitat fragmentation consider abrupt fragmentation events that have occurred within the last decades (e.g. Aldrich and Hamrick 1998, Cosson et al. 1999, White et al. 2002, Quesada et al. 2003, 2004, Baranga 2004, Wieczkowski 2010, Ashworth and Martí 2011, Breed et al. 2012, Noreen and Webb 2013), yet forest fragments may arise as a response to ancient climatic change (Eeley et al. 1999). Given that the montane forests of Africa have experienced periods of small size throughout their evolutionary history (Maley 1996), and given that chloroplast genomes have smaller effective population sizes (Provan et al. 2001, Charlesworth 2009), it is probable that all three species have experienced a similar history of bottlenecks and drift to arrive to contemporary fixation (or near fixation) of chloroplast haplotypes.

Nonetheless, precision of population genetic estimates using cpDNA may be more dependent on the number of populations sampled as opposed to the level of sampling within populations (Petit et al. 2005). Therefore, future work could focus on identifying other populations (of the focal trees species in this study) on the Mambilla Plateau (for example, Kop Nti Forest, ~45km away), and the more distant Gotel mountains (~60km away), to see if they are genetically identical to the Ngel Nyaki populations or whether different genetic variants exist in other populations within this wider region.

Furthermore, the addition of nDNA data, via use of AFLPs or nSSRs, in each tree species could provide interesting insights into how genetic elements with different inheritance patterns have been shaped in Ngel Nyaki—though direct comparisons might not be achieved by these methods. This nuclear genotype data could then be used in parentage analysis of seeds and/or juveniles to compare mating patterns in each of the tree species (allowing estimates of the degree of outcrossing and the extent to which pollen is moved across the forest). In conclusion, if the cpSSR loci used in this study truly reflect chloroplast-wide, or indeed general, genetic patterns it places a sobering thought on the future prospects of these populations and other tree species in Ngel Nyaki (which may be lacking in genetic variance). This study also highlights the need to develop more improved universal microsatellites in plants that provide reliable levels of polymorphism detection for tractable inter-species analysis.

CHAPTER 3

Phylogenetic relationships of West African *Cordia* species

Abstract

Montane areas have been demonstrated to serve as refuges during palaeoclimatic change and as centres for adaptive radiation and species diversity. Genetic tools can help illuminate the amount of diversity harboured in different montane populations by aiding in the identification of distinct evolutionary lineages. Such lineages may be considered as discrete taxa, or in the very least, considered as separate management units. In this study, the taxonomic classification of Ngel Nyaki's resident *Cordia* species was inspected. While previously described as *C. millenii*, morphological observations (not in this study) hinted at the potential distinctness of this population on Mambilla. Additionally, I address broader questions regarding the evolution of the *Cordia* genus with respect to West African taxa. To do so, a combination of cpSSR analysis and phylogenetic analyses (using ITS1, *trnH-psbA*, and *trnL-trnF* sequences) was used. The data suggest that trees identified as *Cordia millenii* on Mambilla are not a unique taxa or evolutionary lineage (based on chloroplast data). The prior hypothesis that all African *Cordia* species belong to a single monophyletic group (the Myxa clade, *sensu* Gottschling et al. 2005) was supported by this study. This work also demonstrates that chloroplast intergenic regions are useful for categorizing *Cordia* into the four general evolutionary lineages in phylogenetic analyses, as outlined by Gottschling et al. (2005), which could prove highly beneficial if the preferred ITS1 sequence is unable to be amplified in difficult samples (though they provide less taxonomic resolution than ITS1).

3.1 Overview

As discussed in SECTION 1.1, the forests of Africa have exhibited expansion and contraction through geological time as a result of climatic change, with montane regions providing refugia for those species that cannot persist under harsher conditions (Hamilton and Taylor 1991, Maley 1996, Elenga et al. 2000, Dupont et al. 2001). While this has led to the surprising homogeneity of vegetation across the Afromontane Archipelago (White 1981), the isolation and reduction in size of populations create a situation where population divergence can occur. For example, populations of *Prunus africana* studied by Kadu et al. (2013), were found to be largely broken into genetically distinct populations delimited by mountain ranges in Africa. At greater extremes, this isolation could drive speciation. Forests on the Mambilla Plateau have likely experienced a history of fragmentation due to historical climatic shifts, but at a more contemporaneous scale this fragmentation has likely been exacerbated by increasing anthropogenic pressure (Chapman and Chapman 2001, Adanu et al. 2010). Thus, gene flow and connection amongst forests on Mambilla has likely decreased consequently over the last decades, but at a broader regional-scale, connectivity has probably fluctuated over evolutionary time.

When populations of organisms become isolated from each other there is a lack of gene flow that normally maintains genetic homogeneity between them; this isolation can lead to the evolution of pre- and post-zygotic barriers, the former relating to obstacles prior to zygote formation (e.g. physical barriers, or differences in ecology), and the latter to obstacles occurring after zygote formation (e.g. genetic incompatibilities, and also ecological disparities; Barraclough and Nee 2001, Schluter 2001). Ecological shifts, vicariance, selection (both natural and sexual), and hybridization are all mechanisms that may drive an ancestral species into two or more distinct taxa (Panhuis et al. 2001, Barraclough and Nee 2001, Schluter 2001, Roberts et al. 2006, Hendry et al. 2007, Niemiller et al. 2008). This process of creating species diversity is speciation (Barraclough and Nee 2001). The rate of speciation itself can vary amongst regions, over time and between taxa (Barraclough and Nee 2001).

Research on birds in montane Africa suggest that following contracted range shifts post-ice-ages, various bird lineages have undergone rapid speciation as a result of their isolation and small populations (Roy 1997, Voelker et al. 2010). Fjeldså and Lovett (1997) demonstrated that patterns of floral and avian richness in tropical Africa suggest that montane areas act as stable habitat during climatic change, which facilitates the protection of relict species and adaptive radiations. During periods of warmer humidity, species can then move between mountain ranges. In the Neotropics, analysis of morphological traits in *Ithomiola* butterflies demonstrates a case of vertically occurring

speciation, with younger species occurring at greater altitudes than those in the lowlands (Hall 2005). Roberts et al. (2006) also found that highland *Epipedobates* frogs in Peru are likely derived from a single ancestral colonization event from the lowlands followed by rapid adaptive radiations, resulting in a greater diversity of more brightly coloured frogs in highlands relative to lowlands. Taken together, these studies support the notion that lowland forests generally harbor older lineages and/or accumulate species over time (i.e. ‘museums’); in contrast, montane reaches and forest at the peripheral edges of lowland habitat act as sources of endemism and speciation (i.e. ‘species generators’).

The importance of understanding biodiversity in conservation has been highlighted by Kahindo et al. (2007) in their review on literature surrounding the genetic relationships of montane bird species complexes. They point out that while under the biological species concept a single taxon may be recognized, application of a phylogenetic species concept using genetic data may actually unearth a number of distinct evolutionary lineages, each potentially worthy of being an individual taxon. Consequently, distinct lineages confined to specific mountains are smaller and more endemic than the species complex as a whole, and therefore should be of particular interest for conservation efforts. Such populations represent separate evolutionary units and need to be managed separately (Frankham 2010). For example, analysis of the mitogenome of North American fishers (*Martes pennant*) across their geographical range revealed at least three distinct lineages, each of which should be managed independently to conserve the genetic integrity of each population (Knaus et al. 2011).

Molecular phylogenetic analysis requires DNA or protein sequence data (Baldauf 2003). It is a process of constructing evolutionary relationships using changes in character states to understand which taxa are most closely related to each other (Barracough and Nee 2001, Baldauf 2003). Construction of a molecular phylogenetic tree begins by assembling a data set of the sequences of interest; next, sequences are aligned; from this multispecies alignment, evolutionary relationships are inferred on differences amongst sequences (Baldauf 2003). To obtain an accurate estimate of the true phylogeny it is important to sample from several individuals per species (especially if assessing relationships amongst closely related populations; Degnan and Rosenberg 2009) and it is important to try and sample as many species from the group of interest as possible (because a phylogeny is only as good as the data set compiled; Barracough and Nee 2001, Baldauf 2003).

Choice of loci to use should be carefully considered. This is because different genes have different evolutionary histories and the relationship that is observed amongst genes may not necessarily reflect the true relationship amongst species; i.e. a disparity between gene and species

trees (Degnan and Rosenberg 2009). Furthermore, the locus of choice needs a rate of evolution rapid enough to generate differences amongst populations, but not so rapid that these relationships become hard to resolve (Barracough and Nee 2001). Organelle genomes commonly have a smaller effective population size than that of the nuclear genome and are thus influenced more strongly by drift (Provan et al. 2001, Ballard and Whitlock 2004). General lack of recombination and uniparental inheritance of organelle genomes means that evolutionary processes affecting one locus will have chromosomal consequences, though there are exceptions (Provan et al. 2001, Ballard and Whitlock 2004; see SECTION 2.1).

As discussed in SECTION 2.1, the different genomes that comprise a plant's complete genetic makeup have their own unique characteristics. While DNA can be obtained from the nucleus (n), chloroplast (cp), and mitochondria (mt), only nDNA is biparentally inherited; also, the slow substitution rate and the presence of recombination in plant mitogenomes means that mtDNA can be difficult to work with (Whitlock and McCauley 1999, Provan et al. 2001, Ballard and Whitlock 2004, Finkeldey et al. 2010).

For phylogenetic analysis it is important to sample markers with different modes of inheritance to understand demarcation of species and evolutionary processes (Barracough and Nee 2001, Li et al. 2011). Li et al. (2011) explored the use of common cpDNA and nDNA markers to identify species, via DNA barcodes, for a large plant data set that comprised a broad range of seed plants across many taxonomic levels. The ability for single, or a combination of, markers to discriminate amongst species was assessed using PWG-Distance, Distance, BLAST, and Tree-Building methods. Results demonstrated that for a two marker combination (with one nDNA and cpDNA marker), the greatest power to differentiate the relationship amongst species was found using the nuclear ITS1 region and the chloroplast *trnH-psbA* intergenic spacer. This marker combination has received support elsewhere (Kress et al. 2005).

Genetic studies of any kind on African members of the subfamily Cordiaceae (family: Boraginaceae) are rare and none have been conducted on this study's focal species, *Cordia millenii* (see Loha et al. 2006, 2009, Derero et al. 2011). The taxonomic richness of Cordiaceae is greatest in the Neotropics (>250 species), which is believed to be the origination of the *Cordia* genus, and there is lower taxonomic richness throughout the Old World (>50 species; Gottschling et al. 2004). Typically, classification of Cordiaceae has been difficult because there are an incredible number of species and huge variances in morphological traits (Gottschling et al. 2005). This was addressed by Gottschling et al. (2005), who reconciled many past taxonomic discrepancies using a combination of ITS1 and *trnL* genetic data and morphological traits. An ITS1 derived phylogeny illustrated two

interesting points: (1) genera *Auxemma*, *Patagonula* and *Saccellium* (other Boraginaceae) are nested within the *Cordia* monophyletic group, and are thus in need of taxonomic reclassification; and (2) *Cordia* species separate out into four well defined clades.

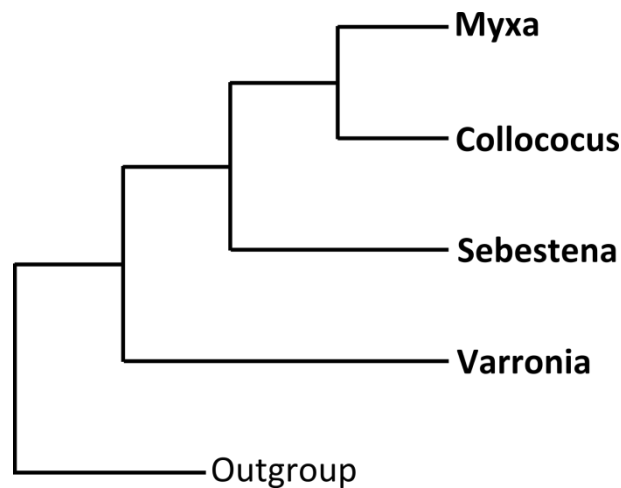


FIGURE 3.1 | Systematic relationships in the Cordiaceae. Cladogram depicts the four distinct clades (Myxa, Collococus, Sebestena, Varronia) that make up this subfamily and the relationships between them (see Gottschling et al. 2005).

Thus, Gottschling et al. (2005) demonstrated the usefulness of resolving taxonomic ambiguity in this subfamily using simple genetic techniques. Briefly, clade Varronia is the sister taxon to all others, clade Sebestena is the sister taxon to clades Myxa and Collococus, which are both sister taxa with respect to each other (FIGURE 3.1). While clade Varronia strictly contains New World species, Myxa contains predominantly Old World species, and Sebestena and Collococus are predominantly New World with some Old World species. The global distribution of *Cordia* taxonomic diversity is believed to be linked to chance trans-continental dispersal events over the genus' evolutionary history (Gottschling et al. 2004, 2005). Using fossils and molecular data, Gottschling et al. (2004) proposed the South American continent to be the origin of *Cordia*, and the ancestors of modern Old World taxa likely arrived to these continents primarily by hydrochoria (water dispersal). This was deemed plausible due to many *Cordia* species possessing seeds with corky endocarps that facilitate hydrochoria (Gottschling et al. 2004).

3.2 Research goals

The purposes of this study were two-fold: (i) determine the distinctness of Ngel Nyaki's *Cordia* relative to those found elsewhere on the Mambilla Plateau and in other parts of West Africa (i.e. is the montane *C. millenii* population at Ngel Nyaki a distinct genetic lineage to those found at other geographic locales?). Collaboration between Kew Royal Botanic Gardens and the Nigerian Montane Forest Project prompted the suggestion that Ngel Nyaki's *Cordia* have somewhat divergent morphology from that currently described for *C. millenii* (leaf and hair traits; pers. comm. Hazel Chapman). This objective thus helps to further assess the importance of Ngel Nyaki Reserve as a hot-spot for biodiversity, and aid conservation in Nigeria, but also West Africa in general. Next, given that Gottschling et al. (2005) only sampled six African *Cordia* (and all of these were either from East Africa, Southern Africa, or Madagascar): (ii) determine whether West African *Cordia* fit with the phylogenetic model proposed by Gottschling et al. (2004, 2005). This second objective will cover gaps in our knowledge on West African biota, which have a tendency to be neglected in the scientific literature (Norris et al. 2010).

3.3 Methods

3.3.1 Sample collection and lab work

C. millenii specimens from Ngel Nyaki were collected as cambium or leaf tissue samples—see SECTION 2.3. Samples of other West African *Cordia* were obtained as herbarium specimens from The Royal Botanic Gardens, Kew; these were received as aliquots of DNA extracts. A list of herbarium specimens can be found in TABLE 3.1. As discussed in SECTION 3.2, the combination of ITS1 and *trnH-psbA* intergenic regions is useful for establishing the relationships amongst species. For this reason, it was decided that these loci should be implemented in this study. However, it was impossible to isolate a clean ITS1 sequence from the herbarium sample MIL31805. Attempts to isolate a complete sequence involved trialing different PCR conditions and primer sets, and when these were unsuccessful, effort was made to try and sequence the ITS2 region as an ersatz locus—but MIL31805 also failed to amplify cleanly in this region. Because of this, an additional chloroplast locus, *trnL-trnF* was added to the data set. Details of primers are listed in TABLE 3.2.

TABLE 3.1 | *Cordia* samples obtained from Kew Royal Botanic Gardens. Species, their original sampling dates and locales, and their specimen identification number are listed.

Species	Date collected	DNA bank No.	Location
<i>C. africana</i>	1968	31789	Nigeria
<i>C. africana</i>	1971	31790	Nigeria
<i>C. aurantiaca</i>	1964	31803	Cameroon
<i>C. millenii</i>	1978	31795	Nigeria, Mambilla Plateau, Akwaizantar Forest
<i>C. millenii</i>	1914	31805	Congo
<i>C. platythyrsa</i>	1964	31797	Nigeria
<i>C. platythyrsa</i>	1965	31806	Cameroon
<i>C. senegalensis</i>	1996	31786	Ghana

TABLE 3.2 | Primer details. Annealing temperature (T_a) for fresh Ngel Nyaki extractions and herbarium extractions (respectively) for ITS1 and *trnH-psbA* differed, as indicated by the “/”. Primer name as per their source.

Locus	Primer name	Primer sequence	T_a	Source
ITS1	P1	5'-TTCAACGAGGAATTCCTAGT-3'	56/55°C	Diane et al. (2002)
	P2	5'-TACGTTCTTCATCGATGCGA-3'		
<i>trnH-psbA</i>	<i>trnH</i>	5'-ACTGCCTTGATCCACTTGGC-3'	58/55°C	Hamilton (1999)
	<i>psbA</i>	5'-CGAAGCTCCATCTACAAATGG-3'		
<i>trnL-trnF</i>	e	5'-GGTTCAAGTCCCTCTATCCC-3'	54°C	Taberlet et al. (1991)
	f	5'-ATTTGAACTGGTGACACGAG-3'		

For *C. millenii* samples from Ngel Nyaki, DNA extraction and PCR mixes were as that described in SECTION 2.3.1. In all but a few cases, Ngel Nyaki samples could be amplified using 20–25µL reaction volumes, but in a few cases this was scaled up to 50uL. PCR conditions for Ngel Nyaki samples were: 3 minutes at 95°C (initial denaturing); 40 cycles of 20 seconds at 95°C

(denaturing), 15 seconds at 56/58/54°C (annealing ITS1/*trnH-psbA/trnL-trnF*, respectively), and 15 seconds at 72°C (extension); completed with 30 seconds at 72°C (final extension). Herbarium samples of West Africa *Cordia* mostly amplified at 25µL reaction volumes. For recalcitrant samples, the troubleshooting guide provided by Kapa Biosystems was consulted and reaction chemistry was adjusted as per following: 50% KAPA Plant PCR Buffer (2×), 0.4µM each primer, 1.5µL DNA, 0.5µL KAPA3G Plant DNA Polymerase, plus the addition of 0.5mM MgCl₂ and the KAPA Enhancer solution at 1× concentration, per reaction (remaining volume made up with PCR water). For herbarium samples, most reactions for all loci produced product with the same PCR conditions as mentioned above for the Ngel Nyaki *Cordia*; the only change being a reduction in the annealing temperatures because it was unclear whether optimized conditions for Ngel Nyaki *Cordia* would work in different populations/species.

All PCR products were run on a 1% agarose gel to ensure successful amplification had taken place and to be sure reactions were free of contaminants. Successful amplifications were then cleaned using AcroPrep™ Plates and run through a second sequencing PCR, in which each forward and reversed strand was amplified individually using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). Sequencing PCR was carried out in a 10uL volume with the following reagent composition: 0.5µL BigDye® Terminator, 1.75µL Sequencing Buffer (5×), 0.32µM primer (forward or reverse), up to 10ng DNA template, and the rest PCR grade water, per reaction. The conditions for this PCR were as follows: 10 seconds at 96°C (denaturing); 25 cycles of 10 seconds at 50°C (annealing) and 1 minute at 60°C (extension).

Following sequencing PCR, the products were cleaned using Sephadex G-50 Fine DNA Grade (GE Health Care). The Sephadex resin was combined with enough MilliQ water so the meniscus of the water was ~1cm above the resin when the solution was allowed to settle. The solution was then mixed to re-suspend the Sephadex and 750µL of solution was transferred to wells in a UNIFILTER® 800 (Whatman®) plate. The plate was then spun for 5 minutes at 750rpm, the spun off water was discarded, and the plate was further spun for as many 2 minute rounds as was required (at 750rpm) until the volume of water in the catchment plate was <5µL. The sequencing PCR reactions were then combined with 10µL MilliQ water (for a total volume of 20µL) and were loaded onto the UNIFILTER plate. The plate was spun at 750rpm for 2 minutes, and this was repeated until ~20µL of liquid passed through the Sephadex. This product was then sequenced on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

In addition to sequence data, cpSSR (chloroplast simple sequence repeat) loci were also used to assess patterns of differentiation between *C. millenii* found on the Mambilla Plateau (Ngel Nyaki,

and MIL31795 from Akwaizantar Forest) and populations found in the Congo. Genotyping of MIL31805 (Congo) was carried out as described in Section 2.3.1. The average genetic distance (\bar{D}_{SH}^2 , *sensu* Vendramin et al. 1998) between identified haplotypes was calculated using EQUATION 2.3 (see SECTION 2.3.2).

3.3.2 Phylogenetic analyses

Each raw sequence (forward and reverse) was inspected individually in FinchTV (Geospiza 2013). Ambiguous bases were identified and sequence files for each read were produced. Some reads were very poor in one, or both directions, which required re-sequencing. Preliminary sequence alignments were conducted in BioEdit (Hall 2013) to identify potential conflict between forward and reverse sequences from the same individual. Any conflicts were resolved by checking the chromatogram for each pair of sequences to resolve a particular base's true identity; if this could not be done the base was marked as ambiguous using IUPAC notation. Within BioEdit, a single consensus sequence was created for Ngel Nyaki and herbarium samples.

Sequences were imported into the genetics program MEGA6 (Tamura et al. 2013) for multispecies alignment, using the MUSCLE (Edgar 2004) package. Details of the alignments are as follows: gap open = -400, gap extended = 0, max iterations = 100, clustering method = neighbor joining, and $\lambda = 24$. For all loci, West African herbarium samples and the Ngel Nyaki *Cordia* were compared to each other but also to *C. panamensis* and *C. alliadora*. Sequences for these latter two species were sourced from GenBank (TABLE 3.3), and the reverse compliment of *trnH-psbA* sequences for both the *C. panamensis* and *C. alliadora* accession was required for the alignments (as their reported sequence was in the wrong direction for this study). Based on the work of Gottschling et al. (2005), it was predicted that West African *Cordia* should fall into the Myxa clade identified by these researchers. Therefore, the choice of including *C. panamensis* (which is in the sister clade, Collococcus, to Myxa) and *C. alliadora* (which is the sister clade, Sebestena, to both Myxa and Collococcus, and thus acts as effective outgroup) provides context to the phylogenetic relationships observed.

TABLE 3.3 | Accession numbers for GenBank sequences. Sequences isolated in this study are listed with a “*”. At the time of this present thesis’ submission, sequences were still under review by GenBank annotation staff and were not available online.

Specimen	Code	Locus	Accession No.
<i>C. africana</i> *	AFR31789	ITS1	KM052599
	AFR31790	ITS1	KM052600
	AFR	<i>trnH-psbA</i>	KM052606
	AFR	<i>trnL-trnF</i>	KM052611
<i>C. alliadora</i>	Alliodora	ITS1	AY321594
	Alliodora	<i>trnH-psbA</i>	JF427964
	Alliodora	<i>trnL-trnF</i>	EU861992
<i>C. aurantiaca</i> *	AUR31803	ITS1	KM052601
	AUR31803	<i>trnH-psbA</i>	KM052607
	AUR31803	<i>trnL-trnF</i>	KM052612
<i>C. millenii</i> *	MIL	ITS1	KM052602
	MIL	<i>trnH-psbA</i>	KM052608
	MIL	<i>trnL-trnF</i>	KM052613
<i>C. panamensis</i>	Panamensis	ITS1	AY321609
	Panamensis	<i>trnH-psbA</i>	JF427921
	Panamensis	<i>trnL-trnF</i>	EU862011
<i>C. platythyrsa</i> *	PLA31796	ITS1	KM052603
	PLA31806	ITS1	KM052604
	PLA	<i>trnH-psbA</i>	KM052609
	PLA	<i>trnL-trnF</i>	KM052614
<i>C. senegalensis</i> *	SEN31786	ITS1	KM052605
	SEN	<i>trnH-psbA</i>	KM052610
	SEN	<i>trnL-trnF</i>	KM052615

Following this, the alignment was imported into BioEdit and sequences trimmed to the length of the shortest sequence to eliminate overhangs (here on referred to as the “original alignment”). The choice of whether or not to keep or remove gaps in the alignment was questioned, as it is recommended that poorly aligned characters and gaps be removed for improving phylogenetic analysis (Baldauf 2003). Initially, Gblocks (Castresana 2000) was used to select unambiguously aligned blocks in the alignment to be kept for downstream analysis. Because these alignments were short (<400bp), less strict Gblock pruning was used to prevent excessive loss of sequence

information, as suggested by Talavera and Castresana (2007). These Gblock conditions were the allowance of (i) smaller final blocks, (ii) gaps in the final alignment, and (iii) less strict flanking positions. The official Gblock server (Institut de Biologia Evolutiva 2014) was used to conduct the analysis.

Preliminary phylogenetic analyses were conducted to determine whether or not subjecting alignments to Gblocks pruning improved the quality of trees produced. As observed in the simulation study by Talavera and Castresana (2007), removal of ambiguous positions produced trees with slightly clearer topologies, but with poorer bootstrap values. Such a phenomenon could be caused by divergent regions creating a bias in the tree's topology that increases bootstrap support for regions where the relationship amongst sequences is uncertain (Talavera and Castresana 2007). Because of this, it is suggested that alignments retaining problematic divergent regions cannot rely on output bootstraps to support their topology (Talavera and Castresana 2007). However, pruning of short sequences can lead to a detrimental loss of information (Talavera and Castresana 2007). This was particularly evident when visually inspecting the blocks designated by Gblocks: some indel sites that could have been particularly diagnostic in discriminating amongst species were removed by Gblocks, even under relaxed conditions.

After considering the above, it was decided that the final analysis would be conducted on unpruned alignments, as sequences were too short to be deemed tractable to Gblocks pruning. Furthermore, alignment gaps can represent phylogenetically informative characters; however, phylogenetic analyses have often regarded each gap position as a different character state, when in fact adjacent gap positions can be viewed as a single character (Simmons and Ochoterena 2000). Therefore, the gap coding software FastGap 1.2 (Borchsenius 2009) was used to code gaps according to the simple indel coding method described in Simmons and Ochoterena (2000). This method codes gaps—regardless of size—as single character states, and creates a data matrix representing the presence and absence of these gaps in the different sequences. Gaps that could be subsets of other gaps are labeled as inapplicable for coding (see FIGURE 3.2 for an explanation).

(a)

Seq. A	AA-- 1 --CGCTT----- 3 -----GG
Seq. B	AA-- 1 --CGCTT----- 3 -----GG
Seq. C	AAT--- 2 ---TT-- 4 --GG- 5 -GGG
Seq. D	AAT--- 2 ---TT-- 4 --GG- 5 -GGG
Seq. E	AATTGCGCGCTTTAACCGGTCAGGG

(b)

Gaps :	1	2	3	4	5
Seq. A	1	0	1	-	-
Seq. B	1	0	1	-	-
Seq. C	0	1	0	1	1
Seq. D	0	1	0	1	1
Seq. E	0	0	0	0	0

FIGURE 3.2 | The simple indel coding method (Simmons and Ochoterena 2000). (a)

The sequence alignment between sequences A–E with numbered gaps highlighted in bold. (b) A coded gap matrix from the exemplar alignment where gaps are present ‘1’, absent ‘0’, or inapplicable ‘-’. Sequences A and B both share gap 1 and gap 3; sequences C and D both share gaps 2, 4 and 5; it is not possible to tell whether gap 3 arose as a subset of gaps 4 and 5, so these gaps are labeled inapplicable for sequences A and B; sequence E has no gaps.

Because phylogenetic trees are never observed (but are instead inferred from sequence information), and because different phylogenetic methods have different strengths, weaknesses, and underlying assumptions, it is important to consider and compare trees built from different methods to see if the inference they provide are comparable (Yang and Rannala 2012). The two methods used here were maximum parsimony and Bayesian inference, both of which are character-based methods in that they simultaneously compare all sequences one site at a time to produce a tree with the highest “score” (Yang and Rannala 2012). In the case of maximum parsimony this is the

minimum number of changes, whilst for Bayesian inference it is the posterior probabilities (Yang and Rannala 2012).

A major strength of maximum parsimony is that it is based on simple assumptions and is easy to comprehend and interpret; however, this simplicity also means that complex information (e.g. the incorporation of knowledge regarding sequence evolution) cannot be included (Yang and Rannala 2012). Bayesian inference benefits from its ability to consistently and efficiently converge on the true tree, as well as the ability to incorporate many different variables into the phylogenetic model; furthermore, posterior probabilities at nodes provide an easy to interpret measure of the trees credibility, representing the likelihood of the relationships given the data and the prior distribution (Yang and Rannala 2012). At the same time, large data sets with complex models can be computationally demanding, which can become a drawback in Bayesian analyses (Yang and Rannala 2012).

Maximum parsimony analysis was carried out in PHYLIP v3.695 (Felsenstein 1989). PHYLIP comes as a package of multiple executable files that are used to carry out each step of phylogenetic tree construction: “infile” for an initial step are subjected to a program that produces an “outfile”, which then becomes the infile for the next step and so on until the final result is obtained. Before alignments were analyzed, “-” gap positions in the original alignment were replaced with “?”. The gap scores matrix coded by FastGap was then appended to the end of the alignment: again, “-” were replaced with “?”, and the binary scoring system for gap presence/absence was replaced with “C” = “1” and “A” = “0” (referred to as the “gap coded alignment”), which were arbitrarily chosen to simply represent character states that could be recognized by PHYLIP.

Separate parsimony trees were constructed as follows (default settings were used unless stated otherwise): (1) the gap coded alignment was input into `seqboot.exe` to create 1,000 bootstrap replicates; (2) resulting bootstrap replicates were then input into `dnapars.exe` (parsimony; analyzing 1,000 data sets with `jumble = 10`); (3) resulting bootstrap trees were then input into `consense.exe` to produce a consensus tree; (4) the consensus tree was unrooted in `retree.exe`, using the “U” subchoice in the “W” choice to write trees; (5) the alignment was then reopened in `dnapars.exe` and option “U” was invoked to “use user trees in input file”, in which the unrooted consensus tree was input to act as the basis of the topology for the inferred sequence tree; (5) the final tree was edited in FigTree v1.4.0 (Rambaut 2007), and *C. alliodora* was made as the root.

Before tree building by Bayesian inference took place, the base substitution model for the original ITS1, *trnH-psbA*, and *trnL-trnF* alignments were estimated using jModelTest v2.1.5

(Posada 2008, Darriba et al. 2012). All default parameters were used, except “Best” was selected for the “Base tree” option, and AIC selection was used to pick the best the model. Bayesian inference was then conducted using MrBayes v3.2.2 (Huelsenbeck and Ronquist 2001). The data was partitioned as either: (1) sequence alignments, which were analyzed as DNA data; and (2) the gap coded matrix, which was analyzed as restriction (binary) data. In MrBayes, gaps and missing data contribute nothing to the phylogenetic analysis, so unlike the parsimony analyses, no “?” needed to be added to the data. Because some models in jModelTest cannot be explicitly coded for in MrBayes, alternative models that most closely approximate these were used instead (*sensu* Vilstrup et al. 2013; see TABLE 3.4).

TABLE 3.4 | jModel Test best substitution models for each locus. NOTE: uf = unequal frequencies of bases; G = rate variation amongst sites; TPM = the K81 model (Kimura 1981); TIM = the transitional model (Posada 2003). The HKY and GTR models were coded for in MrBayes *sensu* Vilstrup et al. (2013), because these are the closest approximation available in MrBayes. All models assert proportion of each nucleotide base is imbalanced.

Locus	jModel Test best model	AIC	According to MrBayes
<i>trnH-psbA</i>	TPM1uf	958.76	HKY
<i>trnL-trnF</i>	TPM1uf	1203.17	HKY
ITS1	TIM1+G	1403.67	GTR+G

3.4 Results

Following trimming of alignments, sequence length was 238 bases for ITS1, 240 bases for *trnH-psbA*, and 350 bases for *trnL-trnF*. Of the 14 gaps observed in the ITS1 alignment, only six were phylogenetically informative, compared to two out of nine gaps for *trnH-psbA*, and two out of seven gaps for *trnL-trnF*. In regard to sequence similarity, all specimens within a single species were identical at both chloroplast loci, but were different at ITS1. The exception to this was between Ngel Nyaki *C. millenii* and MIL31795, where ITS1 sequences were also identical, but given they were samples from the same geographic region this is not surprising; it was impossible to obtain an ITS1 sequence for MIL31805.

Maximum parsimony demonstrated that *trnH-psbA* was better at discriminating amongst taxa than *trnL-trnF*, though only slightly (FIGURE 3.3). The *trnL-trnF* region was not variable enough to resolve the relationships amongst any of the West African *Cordia* species, whilst *trnH-psbA* recognized *C. millenii*, *platythyrsa* and *senegalensis* (MIL/PLA/SEN) as a subclade nested within a larger grouping that includes *C. africana* and *aurantiaca* (AFR/AUR), a two nested clade structure. Because of these results, *trnH-psbA* and *trnL-trnF* were combined for all further analyses as chloroplast loci. The combination of the two chloroplast intergenic regions (FIGURE 3.4) partially increased the support for the relationship amongst taxa with maximum parsimony, which identified a weakly supported (bootstraps <0.50) sister relationship of *C. aurantiaca* to the other West African *Cordia*, and a sister relationship of *C. africana* to the MIL/PLA/SEN grouping (a three nested clade structure). The Bayesian tree did not observe this, probably because posterior probabilities for this relationship were <0.50 and not represented in the final consensus.

Phylogenies of ITS1 (FIGURE 3.5) also weakly supported the sister relationship of *C. aurantiaca* to all West African *Cordia* in this study. Maximum parsimony also weakly supported a further separation of *C. senegalensis* sister to *C. platythyrsa* and *millenii*, which are sister to each other (a four nested clade structure). Bayesian inference of ITS1 only supported a three nested clade structure of West African *Cordia*. When ITS1 data was combined with chloroplast data (FIGURE 3.6), support for the four nested clade structure barely changed for maximum parsimony analysis and the Bayesian inference tree was collapsed to the simpler two clade nested structure.

In all cases—regardless of locus, the combination of loci, or the tree building method—*C. panamensis* was always found sister to the West African *Cordia*, and the closest relative to *C. alliodora* out of the focal species. This result reflects the phylogenetic relationship predicted by Gottschling et al. (2005) based on ITS1 data. It demonstrates that West African *Cordia* also belong to the more recently derived Myxa clade (which constitutes *Cordia* species from other parts of Africa).

Inspection of *ccmp* genotypes indicated that the Nigerian populations of *C. millenii* on the Mambilla Plateau have different, but very similar, haplotypes to those in the Congo. The defining difference between these regions was at *ccmp2*: in Mambilla, this locus has a 185bp allele, whilst the Congo population is in a 186 bp state. Alleles at the other six loci are as follows: *ccmp3* = 106bp; *ccmp4* = 127bp; *ccmp5* = 101bp; *ccmp6* = 70bp; *ccmp7* = 125bp; and *ccmp10* = 109bp. The \bar{D}_{SH}^2 was estimated to be 0.14bp between Congo and Mambilla populations, equating to the 1bp difference averaged across seven loci.

3.5 Discussion

The objective of this study was to understand how isolation of a population of *C. millenii* in a montane region of Nigeria may have contributed to divergence and formation of a distinct genetic lineage (with respect to those in the same region and in different regions), as well as further elaborating on the phylogenetic relationship of West African *Cordia* species with respect to the greater taxonomic picture of the genus' evolution. While the data described here is able to answer the latter question, the former question remains partially unresolved.

The use of phylogenetic methods to resolve taxonomic relationships amongst plants is a morass of opposing opinions. While mitochondrial genes appear very suitable at barcoding the diversity of life in animals, this cannot be said for plants because plant mtDNA evolves much more slowly, and ergo, accrues fewer differences between species (Provan et al. 2001, Pennisi 2007). Because of its abundance in plant cells and its faster evolutionary rate, chloroplast loci are valuable for plant phylogenetic tests (Pennisi 2007, Dong et al. 2012). However, the ability to settle on a truly universal genetic marker for plant taxonomists is made difficult by conflicting needs amongst researchers; while some markers may be fine for more distantly related species, they may be poor discriminators when taxa share younger evolutionary relationships (Pennisi 2007).

Many different cpDNA loci are reported in the literature (Kress et al. 2005, Kress and Erickson 2007, Lahaye et al. 2008, Dong et al. 2012), also popular are the nuclear ITS regions, both alone and in combination with cpDNA (Kress et al. 2005, Yao et al. 2010, Li et al. 2011). The ITS1 region has been demonstrated to be a useful locus in understanding the phylogenetic relationships of members of the Boraginaceae (Gottschling et al. 2004, 2005). Furthermore, studies covering many different plant species across multiple families have demonstrated highly successful taxonomic discriminatory power of combining ITS1 and the intergenic cpDNA *trnH-psbA* sequences (Kress et al. 2005, Li et al. 2011). For these reasons, ITS1 and *trnH-psbA* were initially selected to determine if the *C. millenii* at Ngel Nyaki are genetically distinct to those found elsewhere on the Mambilla Plateau but also in other more distant populations in West Africa.

The notion to assess the taxonomic novelty of Ngel Nyaki's *Cordia* were sparked by the theory that montane regions are believed to be promoters of diversification and because collaboration between Kew Royal Botanic Gardens and the Nigerian Montane Forest Project lead to speculation of Ngel Nyaki's *Cordia* having distinct morphological traits. Morphological analysis of *Ithomiola* butterfly species in the Neotropics suggests that successively younger taxa occur at increasingly higher altitudes relative to their nearest sister taxon, an example of vertical montane speciation (Hall 2005). Other cases where montane environments have been attributed to radiation events are

found in birds, frogs, and plants, and are associated with colonization and isolation processes (Fjeldså and Lovett 1997, Roy 1997, Roberts et al. 2006, Voelker et al. 2010).

The pattern of dispersal during range shifts (like those caused by glacial cycles) will impact the distribution of genetic variation: rapid population shifts will likely lead to increased homozygosity at the expansion front due to founder effects, whilst a slower expansion will allow more alleles to survive and reduce among population divergence (Hewitt 1996). Cycles of climatic events create alternations between allopatry and sympatry, commonness and rarity, and continuous and fragmented distributions for species (Bartlein and Prentice 1989). Given that populations of trees on the Mambilla Plateau make up West Africa's montane forest elements, it is most probable that they have exhibited patterns of coalescence and fragmentation throughout their history (see Maley 1996, Eeley et al. 1999).

While climatically induced range shifts (e.g. like those caused by glacial cycles) can cause populations to genetically diverge, intermittent periods where populations expand and come back into contact may facilitate genetic mixing that resists differentiation if the time spent in allopatry is not sufficient to accrue genetic incompatibilities (Bartlein and Prentice 1989, Knowles 2001, Galbreath et al. 2009, Rubidge et al. 2012). Using COI data from Rocky Mountain populations of the grasshopper *Melanoplus oregonensis*, Knowles (2001) demonstrated that glacial events have driven divergence amongst populations via drift (during colonization post-glaciation) and through isolation (in allopatric refugia during glacial periods). Furthermore, while range expansions and contractions will assort neutral genetic variance across the landscape, varying selection regimes across the species' range will select for different genomic elements in disparate populations, and there is possibly an interplay between species moving to where they are most adapted and species adapting to local conditions (Hewitt 1996).

At greater region-wide scales, genetic patterns can elucidate how glacial events may have driven genetic patterns through historical and contemporary processes (Alexandrino et al. 2000, Knowles 2001). For one, if population divergence comes about due to separation of populations into allopatric refugia during a glacial period, it is expected that there will be some regional structuring in this variance linked to the spatial positioning of populations: for example, European populations of *Fraxinus excelsior* ash underwent significant divergence in their glacial refugium, which has had marked effects on post-glacial spatial pattern of haplotype diversity (Heuertz et al. 2004). On the other hand, divergence can instead be driven by the expansion of populations post-glaciation, leading to founder effects in these younger populations (which lose genetic diversity from

subsampling their source populations): such a pattern in tree populations has been observed in European hornbeams (Grivet and Petit 2003)

Understanding how disconnection amongst populations in a montane setting relates to the genetic characteristics of populations is important for conservation. In their review, Kahindo et al. (2007) champion the use of genetic tools to identify distinct evolutionary lineages for management purposes. The motive behind such a method is that genetic differences can be used to resolve taxonomic ambiguities that might fail to recognize important biodiversity in small, isolated montane populations. This rationale was adopted for this study and results suggest that: (1) based on nDNA and cpDNA data, different forests on the Mambilla Plateau contain *C. millenii* populations of the same lineage; and (2) based on cpDNA alone, the *C. millenii* on the Mambilla Plateau do not represent a distinct genetic lineage from those observed in other regions, but do show some regional structuring of cpSSR genetic diversity.

Sequence data of both intergenic spacers for the Mambilla *C. millenii* and the Congo specimen (MIL31805) were identical. Chloroplast intergenic spacers lie between coding genes, and differ in their variability (Dong et al. 2012). Many lines of evidence suggest that *trnH-psbA* is one of the best choices for delimiting systematic relationships, especially in conjunction with other loci (Kress et al. 2005, Kress and Erickson 2007, Lahaye et al. 2008, Li et al. 2011). In contrast, out of nine cpDNA intergenic spacers examined by Kress et al. (2005), the *trnL-trnF* was found to be the least variable in comparisons of *Atropa* and *Nicotiana* chloroplast genomes. However, plant systematic studies have demonstrated *trnL-trnF* to be useful in resolving phylogenetic relationships (McDade and Moody 1999, Nyffeler 2002). Therefore, genetically speaking, there is a lack of evidence to support a distinctive montane lineage of *C. millenii* on the Mambilla Plateau. However, it should be noted that understanding speciation patterns in trees (especially between closely related sister taxa) can be very complicated due to the thorny relationship between morphological and molecular divergence (Dainou et al. 2014).

Phylogenetic analysis of cpDNA illustrated significantly poorer resolution of taxonomic relationships for *trnL-trnF*, versus *trnH-psbA*, despite a greater amount of nucleotide positions, and there were no sequence differences within species. Overall, there were a greater number of base substitutions in *trnH-psbA* sequences, but a larger proportion of gaps in *trnL-trnF* were informative. Egan and Crandall (2008) proposed that incorporation of gaps as phylogenetically informative data is highly relevant to chloroplast sequence analysis. In the case of this study, gaps were coded using the simple indel coding method (Simmons and Ochoterena 2000), but >30% of all gaps were uninformative. Lack of clade structuring produced by chloroplast data suggests a lack of

apomorphies (derived character states) to confidently separate sequences in each taxon into monophyletic groups (Bremer and Wanntorp 1978). However, support for relationships was improved by concatenating *trnL-trnF* and *trnH-psbA*.

Contrastingly, ITS1 data alone was able to identify greater phylogenetic structuring amongst species: however, the relationships amongst *C. millenii*, *platythyrsa* and *senegalensis* were not strongly resolved, nor the relationship of *C. aurantiaca* and *africana* to this group. Unlike the chloroplast loci, the ITS1 sequences showed variation within a species. Within species sequence divergence was not seen between *C. millenii* from Ngel Nyaki or Akwaizantar, which is unsurprising because they are from the same region (the Mambilla Plateau, Nigeria). It is a pity that an ITS1 sequence was not able to be obtained from MIL31805 (from the Congo), as it would have been useful to compare ITS1 sequence divergence in *C. millenii* relative to other species (*C. platythyrsa* and *africana*) where multiple specimens were available. Combination of ITS1 data with both chloroplast loci did little to improve bootstrap support values of relationships in maximum parsimony, and even detracted from support in Bayesian inference.

It is known from work in *Prunus africana* that regional-wide patterns of variation across Afromontane regions can be driven by isolations and divergence processes between mountains (Kadu et al. 2013). Use of seven cpSSR loci (ccmp primers, Weising and Gardner 1999) in this study demonstrated that *C. millenii* on the Mambilla Plateau share a largely similar haplotype to the Congo specimen (MIL31805). Distinction between the Mambilla and Congo haplotype was due to a 1bp difference at the ccmp2 locus. These results support conclusions that the montane Mambilla *C. millenii* are not a new or distinct genetic lineage. It should be noted that SSRs are prone to homoplasy, which may complicate discernment of taxonomic relationships using such loci (Provan et al. 2001, Selkoe and Toonen 2006). Homoplasy, however, should not be a confounding factor in this present work because it seems unlikely that so many cpSSR loci would converge on the same allele.

Additionally, the data in this study support the hypotheses of Gottschling et al. (2004, 2005): *Cordia* species in Africa are all from the most derived group of *Cordia*, Myxa. In all analyses—regardless of locus or method—all West African *Cordia* clustered together in a single clade, sister to *C. panamensis* (Collococus group). Gottschling et al. (2005) demonstrated that *C. africana* is a member of Myxa, and given that all West African specimens (except *C. aurantiaca*) were more derived than *C. africana*, it is safe to say that *Cordia* from West Africa (studied here) are also within the Myxa group. It is hypothesized that *Cordia* in Africa are derived from ancestors from the Americas, and splitting of New and Old World Myxa species dates back approximately 40 million

years (Gottschling et al. 2004). African members of *Myxa* are believed to be decedents of ancient long-distance dispersal events from the New to Old World, which has created a disjunct transoceanic distribution amongst the clade members (Gottschling et al. 2004, 2005).

To give a précis of this study's results: The *C. millenii* found on the Mambilla Plateau are unlikely distinct genetic lineages to those found at greater region-wide scales—at least not enough to be considered evolutionarily distinct taxa. This is based on sequence data obtained from two cpDNA intergenic spacers (*trnH-psbA* and *trnL-trnF*) and seven cpSSR loci. Regardless of locus or method, phylogenetic relationships amongst the West African species considered and the two outgroup taxa (*C. panamaensis* and *alliodora*) were always consistent. West African *Cordia* fall into the *Myxa* clade as would be predicted from prior work, supporting the hypothesis that all African *Cordia* are derived from a single ancestral lineage from the Americas (Gottschling et al. 2004, 2005). In this study, while there were always two major groupings of West African *Cordia* (MIL/PLA/SEN and AFR/AUR), the resolution of relationships within these groups was mostly reliant on the locus; ITS1 data was most variable and able to better resolve relationships than either chloroplast locus alone or in combination. Difficulty of phylogenetic analyses to tease apart the relationships with considerable confidence could be due to a relatively young divergence times amongst the West African *Cordia* studied, such that the genetic differences between species are comparatively low.

This work demonstrates the need to incorporate different markers in plant systematics. A good phylogenetic marker needs to evolve rapidly, but not too rapidly, so as to make relationships easy to resolve (Barracough and Nee 2001). Unfortunately ITS1 data could not be obtained for every single herbarium specimen, but degradation of old, preserved specimens makes many steps of sequence acquisition difficult (see Rogers and Bendich 1985, Blattner 1999). The general lack of clarity in cpDNA derived trees is attributed to the homogeneity of sequences of each species from different regions, likely the product of the slower substitution rate in the chloroplast (versus nuclear) genome (Muse 2000). Despite this, results do suggest general evolutionary relationships in the Cordiaceae can be inferred with cpDNA if the more desirable ITS1 is not able to be isolated (e.g. in highly degraded samples like those from herbaria).

Future work should sample more chloroplast loci and more taxa from a wider range of localities, as this additional data will surely add to resolution power (Barracough and Nee 2001, Baldauf 2003). Furthermore, this study has contributed to a better understanding of the systematic relationships of African *Cordia*, particularly those from West Africa. While a considerable amount of genetic work has been done on *Cordia* native to the Americas, such a rich database is lacking for

African species. Data from this study will therefore be valuable to future plant systematicists in search of African *Cordia* sequences. Finally, this study used molecular data to answer a question sparked by observations of morphological traits in regards to the taxonomic uniqueness of Ngel Nyaki's *Cordia*. While the data here suggests a lack of taxonomic distinctness, greater in depth examination of morphology might elucidate more concrete character differences between Ngel Nyaki's *C. millenii* and those found elsewhere in Africa. Such work would prove interesting in understanding how different montane populations diverge morphologically in response to different environmental conditions and how this correlates with genetic divergence.

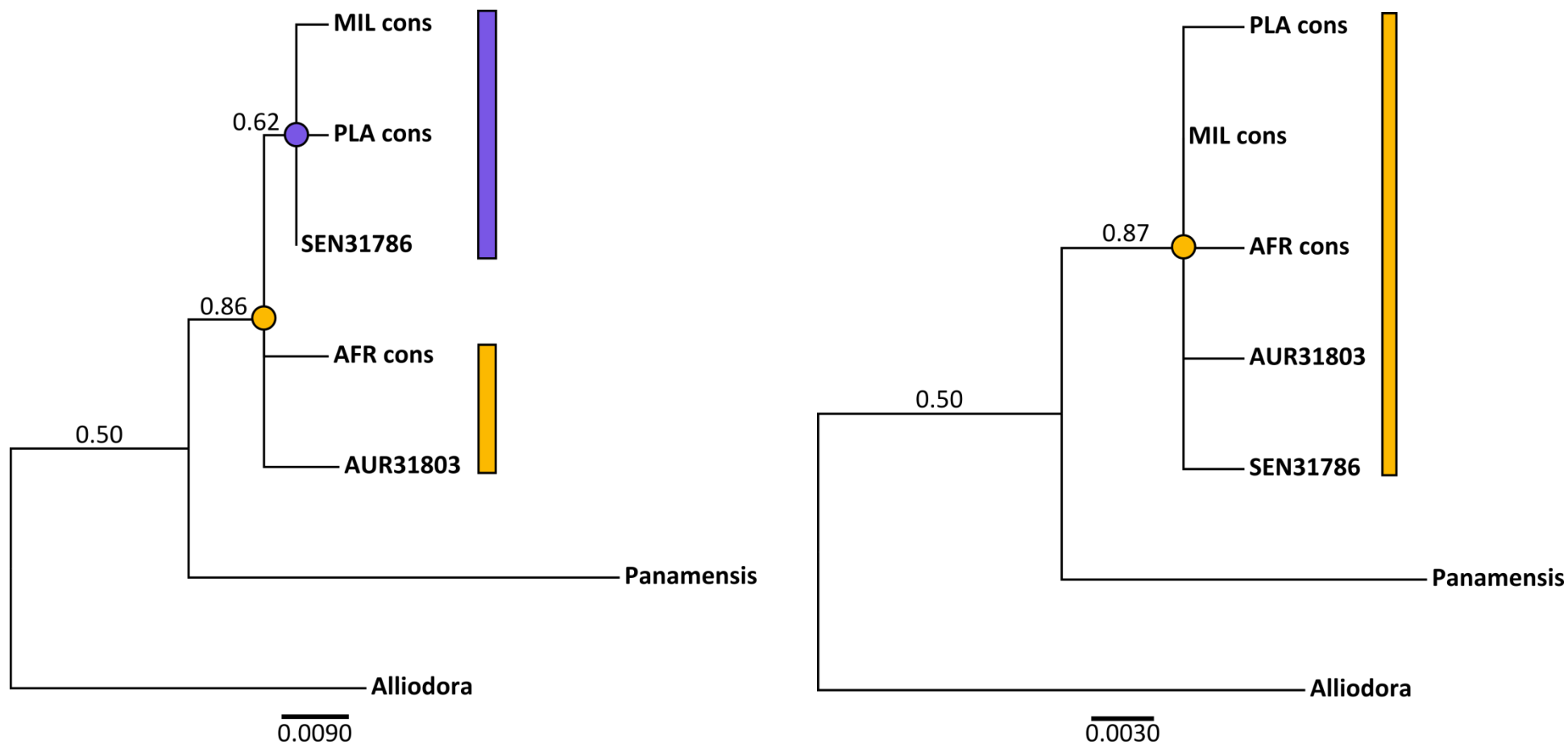


FIGURE 3.3 | Maximum parsimony trees for *trnH-psbA* (left) and *trnL-trnF* (right). Only bootstrap values ≥ 0.50 are shown. Identical sequences of specimens within a species are represented as single consensus (“cons”). Coloured bars and circles indicate key phylogenetic relationships observed between species. From the bottom up, groupings amongst species become increasingly more derived and nested.

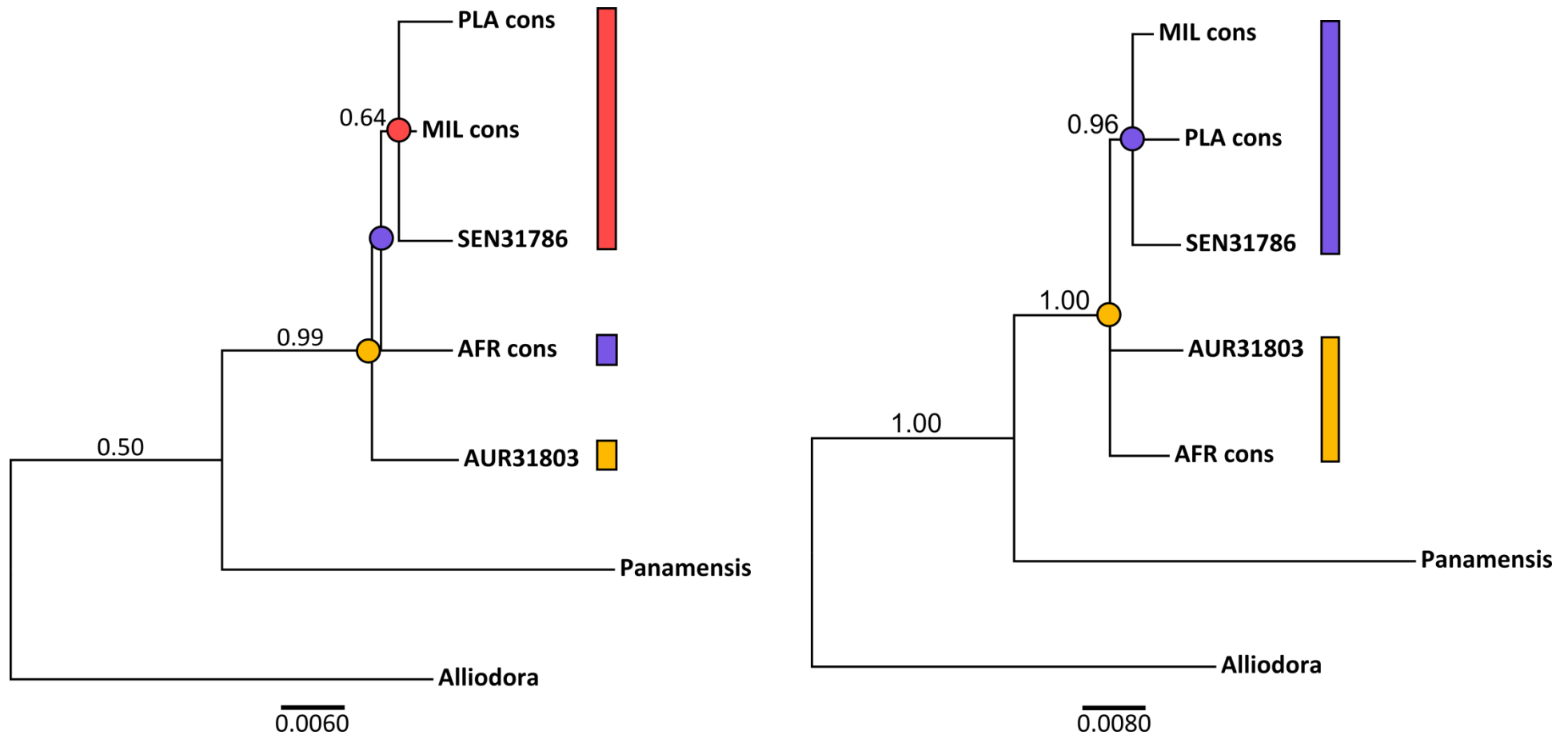


FIGURE 3.4 | Chloroplast loci (combined sequences for *trnH-psbA* and *trnL-trnF*) maximum parsimony (left) and Bayesian (right) trees. For maximum parsimony only bootstrap values ≥ 0.50 are shown, and for Bayesian inference branch labels indicate the posterior probabilities. Coloured bars and circles indicate key phylogenetic relationships observed between species. From the bottom up, groupings amongst species become increasingly more derived and nested.

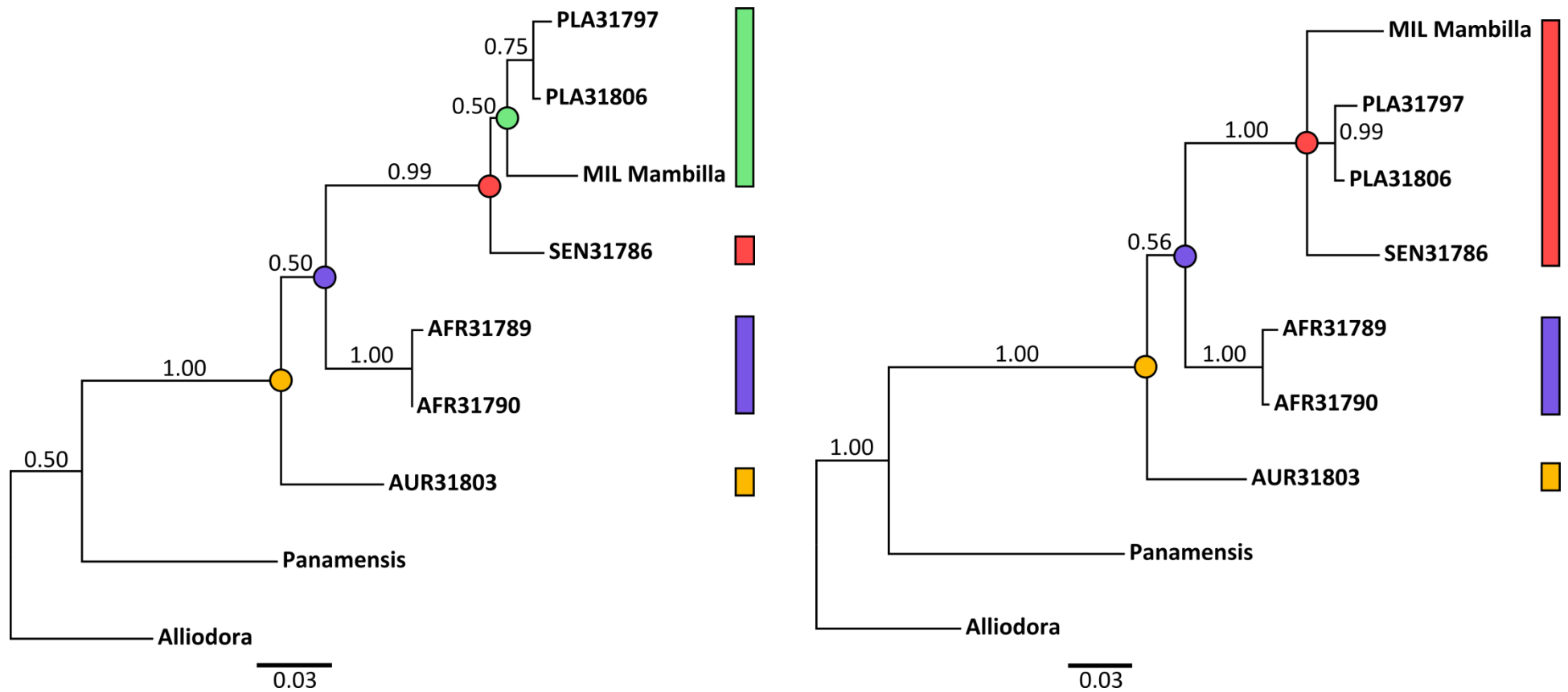


FIGURE 3.5 | ITS1 maximum parsimony (left) and Bayesian (right) trees. For maximum parsimony only bootstrap values ≥ 0.50 are shown, and for Bayesian inference branch labels indicate the posterior probabilities. Lack of ITS1 sequence data for MIL31805 meant only *C. millenii* from the Mambilla Plateau (“Mambilla”) were used to construct these trees. Coloured bars and circles indicate key phylogenetic relationships observed between species. From the bottom up, groupings amongst species become increasingly more derived and nested.

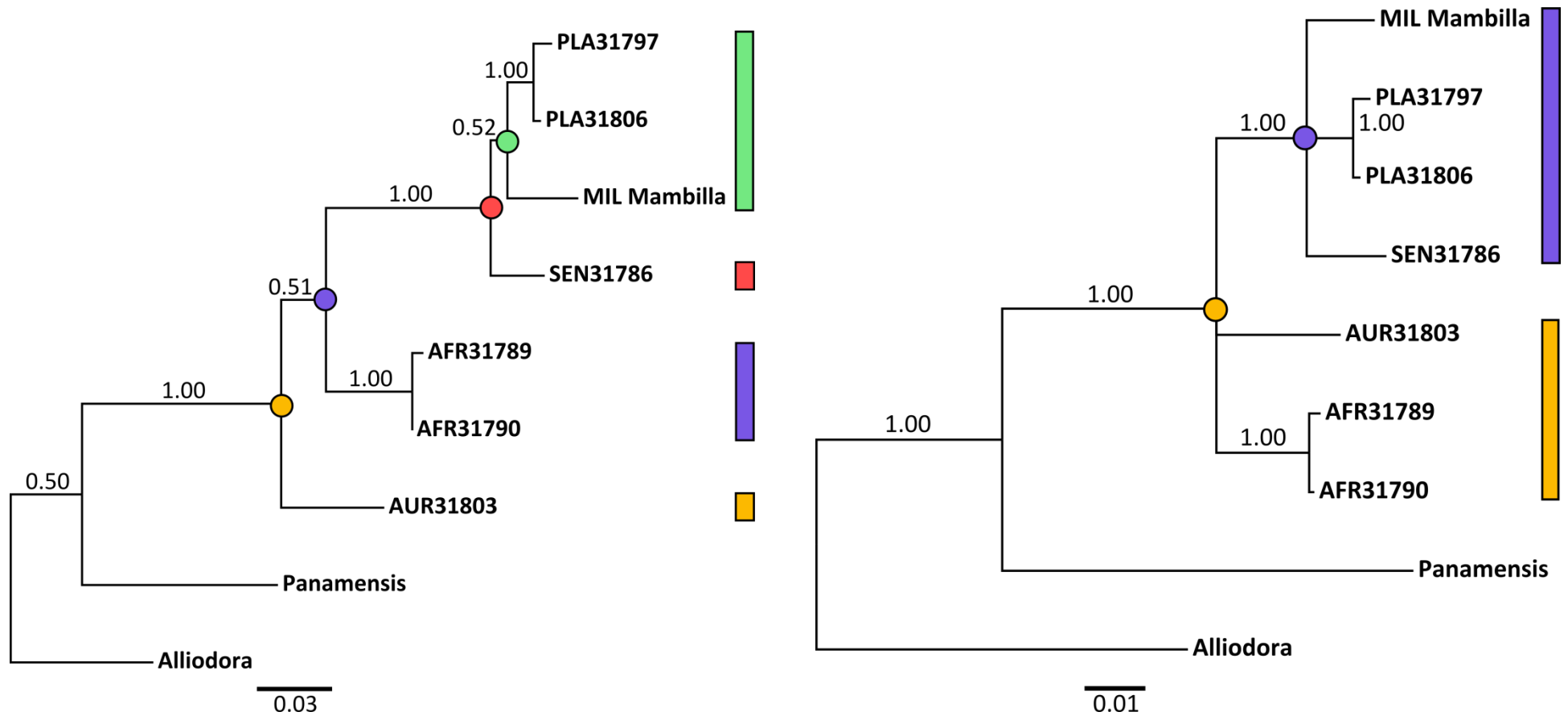


FIGURE 3.6 | ITS1 + chloroplast loci maximum parsimony (left) and Bayesian (right) trees. For maximum parsimony only bootstrap values ≥ 0.50 are shown, and for Bayesian inference branch labels indicate the posterior probabilities. Lack of ITS1 sequence data for MIL31805 meant only *C. millenii* from the Mambilla Plateau (“Mambilla”) were used to construct these trees. Coloured bars and circles indicate key phylogenetic relationships observed between species. From the bottom up, groupings amongst species become increasingly more derived and nested.

CHAPTER 4

Regeneration of montane forest trees

Abstract

Successful recruitment of tree populations in disturbed forest can be constrained when anthropogenic activity impacts important biotic and abiotic factors necessary for the establishment of seeds to adult trees. Ngel Nyaki Forest is an example where fire, defaunation (via hunting), and cattle grazing threatens the forest's regeneration. Initially, three tree species (*Cordia millenii*, *Entandrophragma angolense*, *Lovoa trichilioides*) were selected to undergo a juvenile population survey to assess how different tree species might be recruiting within Ngel Nyaki Forest. *C. millenii* was identified as doing incredibly poorly, so a number of experiments were conducted to understand aspects of *C. millenii*'s regeneration ecology at Ngel Nyaki. Seed dispersal assessments demonstrated that if unaided by vertebrate dispersers, *C. millenii* seed fails to disperse far from the crown edge (with a high density of seed directly under the crown). Furthermore, removal of *C. millenii* seed by ground-dwelling vertebrates is very low, suggesting a general absence of secondary dispersal for this species. Finally, transplant and exclusion experiments were used to test the importance of habitat, conspecifics, and vertebrate and invertebrate herbivory as factors that impact *C. millenii* seedling survival. Only habitat proved to be significant: seedlings growing in forested habitat had dramatically lower survival rates than those in grassland, which was attributed to lack of sunlight. Taken together, results suggest that large-seeded, primate-dispersed species like *C. millenii* are likely to exhibit constrained recruitment if local primate populations (particularly chimpanzees) are extirpated due to their specific dispersal agent requirements. Regarding reforestation efforts at Ngel Nyaki, the balance between adequate sunlight and cover to prevent desiccation of *C. millenii* seedlings could be provided by isolated trees in grassland. Isolated trees could be used as nursery sites to establish small groves of trees attractive to frugivores (e.g. *C. millenii*), which could aid greater connection of fragments and movement of seeds by encouraging frugivores to move into the grassland. In the long-term, this may facilitate fragment coalescence and increase the forest cover at Ngel Nyaki Forest Reserve.

4.1 Overview

A diverse range of abiotic and biotic factors impact the specific regeneration strategies of forest tree species. For example, seed germination is influenced by the life-history traits of the species concerned (Garwood 1983, Vázquez-Yánes and Orozco-Segovia 1984, Yu et al. 2008). Seed dormancy strategies are under selection, balancing costs of reproduction, germination and availability of resources (Garwood 1983). Seed species show varying degrees of desiccation tolerance, which is important, because moisture content in a seed is positively related to successful germination (Yu et al. 2008). Seed banks in forests represent communities of dormant seeds waiting for the right conditions to germinate (Vázquez-Yánes and Orozco-Segovia 1984), and this persistence of viable seed in the soil can be on the scale of decades, which may play a role in buffering tree populations from environmental heterogeneity and loss of genetic diversity (Dalling and Brown 2009).

Treefall gaps in the forest are an important phase in a forest's regeneration cycle (Brokaw 1985). Population and size class structure are related to the stage in the regeneration cycle that persists in the gap; while some species wait as juveniles for a gap to open, others wait as seeds in the soil, or are dispersed as seeds into the gap (Brokaw 1985). As outlined in Brokaw (1985), pioneer (shade-intolerant) species generally colonize gaps as seeds, and tend to be animal-dispersed with a high prevalence for dormancy. In contrast juveniles of non-pioneer (shade-tolerant) species are able to germinate under closed canopy, where they persist as suppressed juveniles until an appropriate gap forms. Although mortality in the understorey is high, a number of juveniles will persist until a gap opens, and this requires physiological adaptation and the ability to shift resource allocation to varying levels of shade (Brokaw 1985, Bongers et al. 1988, Popma and Bongers 1988). The ability for seedlings to utilize the additional light for growth, and acclimate to gap formation, may also be dependent on the relative size of the gap, with larger gaps triggering greater response (Popma and Bongers 1991).

Adaptations to specific microhabitats are likely essential for successful establishment of seedlings into reproductive adults (Clark et al. 1993, Rey and Alcántara 2000, Hall et al. 2003, Baraloto et al. 2005, Makana and Thomas 2005). Soil type, light intensity, leaf litter, moisture and forest disturbance are all factors playing important roles in determining survival, growth and success of tree seedlings (Clark et al. 1993, Rey and Alcántara 2000, Hall et al. 2003, Baraloto et al. 2005, Makana and Thomas 2005). The dissimilarity in microhabitat requirements of different species may facilitate the coexistence of multiple species in tropical forests, thus accounting for their high biodiversity (Hall et al. 2003, Baraloto et al. 2005). Additionally, it is interesting to note

that the microhabitat factors that may be beneficial for seeds may be different to that required for seedling survival and establishment, thus requiring changes in the microsite conditions over time for successful recruitment (Rey and Alcántara 2000).

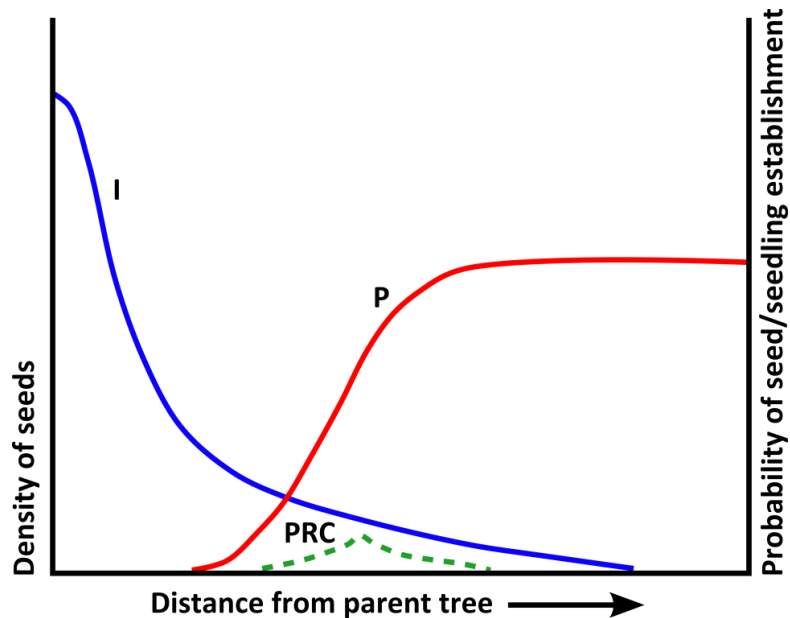


FIGURE 4.1 | A general relationship between number of seeds, probability of seed/seedling establishment, and the distance from parental trees. With increasing distance from the parent the number of seeds per unit area (*I*) declines rapidly (blue line). Contrastingly, the probability that this seed, or its emergent seedling, will survive to maturity (*P*) increases at greater distances from the parent due to declines in host-specific seed and seedling predators and/or pathogens (red line). The product of *I* and *P* gives *PRC* (the population recruitment curve) peaking at some intermediate distance from the parent where dispersal is moderate and successful establishment reasonably probable (green line). SOURCE: modified from Janzen (1970), with permission.

Seed dispersal may be necessary to escape parent-proximity mortality, to colonize new areas, or to take seeds to ideal microhabitats for successful establishment (Schupp 1993). While the patterns of recruitment of wind-dispersed species are largely dependent on direction of prevailing winds, seed shadows produced by animals are complex and variable, and resulting patterns are the product of both the disperser and tree species' biology (Janzen 1988, Wunderle 1997, Russo and Augsperger 2004, Bravo 2008). Dispersal is defined as the quantity (i.e. number of visits to a plant, and number of seeds dispersed) and quality (i.e. quality of seed treatment, and quality of deposition)

of a given disperser's service (Schupp 1993, but see Parrado-Rosselli and Amaya-Espinel 2006). The foraging patterns of frugivores are also correlated to the abundance of fruit spatially and temporally (Blake and Loiselle 1991, Peres et al. 1997, Jorge and Peres 2003, Saracco et al. 2005, Emsens et al. 2013). Success of seed dispersal is imperative because it is a key process in the regeneration cycle of a tree, linking reproduction to growth and establishment (McConkey et al. 2012).

Predation (by mammals and invertebrates) and infection (by pathogenic fungi) are yet another source of mortality for seeds and seedlings (Packer and Clay 2000, Benítez-Malvido and Lemus-Albor 2005, Grogan and Galvão 2006, McKenna and McKenna 2006, Hall 2008). Now a classic piece of theory in tropical ecology, the Janzen-Connell (JC) Hypothesis postulates that mortality of tree recruits is a function of density- or distance-dependent factors due to herbivores and pathogens that aggregate around parental and conspecific trees (Janzen 1970, Connell 1971; FIGURE 4.1). To escape these locally aggregated enemies, seeds need to disperse abiotically (e.g. via wind or water) or by biotic agents (i.e. via animals) away from the parent tree (see Effiom et al. 2013). Studies illustrate that survival under parental trees can be incredibly poor, and thus adequate dispersal of seed is necessary for healthy population regeneration (Rey and Alcántara 2000, Babweteera et al. 2007, Babweteera and Brown 2010, Bagchi et al. 2014).

Human disturbance exacerbates or impacts key factors that may be essential in the recruitment process. Logging alters the physical environment of the forest by killing off reproductive adults, altering the vegetation structure, opening gaps in the forest, and ultimately causing habitat degradation and fragmentation (Curran et al. 1999, discussed in Jansen and Zuidema 2001). Though seedlings can survive and grow in gaps created via logging (Vieira et al. 2007), competition with rapidly colonizing vegetation can limit recruitment (Fredericksen and Mostacedo 2000). Deforestation can also impact the interactions with seed dispersers by altering vertebrate communities and their food sources, which may drive changes in community composition (Jansen and Zuidema 2001, Effiom et al. 2013, Aliyu et al. 2014)

The loss of forest habitat is thus linked to the processes that govern successful regeneration in tree populations. Similarly to tree felling, fire can also be a destructive force that disturbs tropical forests, and a history of previous burning increases a forest's susceptibility to future fire events (Cochrane et al. 1999). Therefore, in areas where human-induced forest fires occur at a greater than natural frequency, there is potential for positive feedbacks of fire that prime the forest for a downward spiral of degradation (Cochrane et al. 1999). Aside from direct destruction of forest, fire

also opens up the edges of forest, allowing greater penetration of edge effects that alter the abiotic microclimates within the forest (Didham and Lawton 1999).

Edge effects are, however, a naturally occurring phenomenon of forests, in which the microclimate within the first few hundred meters of forest is different and more variable than that of the innermost core of the forest (Gascon et al. 2000). Deforestation and subsequent fragmentation ultimately increases the ratio of edge habitat to core forest habitat, meaning that a greater proportion of the forest has abiotic conditions more analogous to an edge environment; such conditions may be hotter temperatures, lower humidity, more intense solar radiation, increased leaf litter, and greater seed predation in fragmented forest (Bruna 1999, 2002, Gascon et al. 2000, Jorge and Howe 2009, Aliyu et al. 2014). Recruitment of tree seedlings and their diversity has been found to decline from the core to edge forest, and environmental stress at edges may enhance damage caused by pathogens and herbivores (Benítez-Malvido and Martínez-Ramos 2003, Benítez-Malvido and Lemus-Albor 2005, Sugiyama and Peterson 2013). Contrastingly, Bouroncle and Finegan (2011) proposed that forests need not “melt down” at their edges provided functional characteristics (like high stem turnover, or low mean seed volume) buffer them from anthropogenic disturbance; it should be noted, however, that their research was for Neotropical, not Afrotropical, forest.

Dispersal of seed at the boundary of forests is essential for the expansion of forests (Janzen 1988), and low availability of seeds can limit regeneration of trees into abandoned grassland pastures (surrounding forest fragments) and in forest gaps (Wijdeven and Kuzee 2000, Holl et al. 2000, Castillo and Stevenson 2010). In addition, trees isolated in pasture can represent important points of regeneration and expansion of forest (Guevara et al. 1986, Janzen 1988). At these isolated trees, animals congregate in search of food, rest and shelter, bringing with them seeds that are deposited under the crown (Berens et al. 2008, Chimera and Drake 2010, Pizo and Santos 2011). Because tropical forest conservation has now inexorably become a process of managing habitat fragments, ecologists working in such habitats need to focus on arresting their ecological decay and promoting their growth and coalescence into larger forest patches (Janzen 1988, Holl et al. 2000).

4.2 Research goals

This chapter aims to address the regeneration of tree populations in Ngel Nyaki Forest. The three species chosen were *Cordia millenii*, *Entandrophragma angolense*, and *Lovoa trichilioides*. These trees are all under logging pressure in Nigeria (Adekunle 2006, Adekunle et al. 2010) and were selected based on differences in conservation status, distribution within Ngel Nyaki, and their ecology (SECTION 1.4.2, TABLE 1.1).

The first question asked was: (i) what does the recruiting juvenile population look like in the three study species? The results from this first question suggested inhibited recruitment of the edge favouring *C. millenii*, and lead me to ask the following questions: (ii) what does the seed shadow of this species look like in Ngel-Nyak; (iii) what is the level of seed predation; and (iv) what factors possibly impact *C. millenii* seedlings' success? Ngel Nyaki is a forest fragment under threat from cattle, hunting and fire encroachment. Conservation of *C. millenii* is important because it is a valuable fruit source to primates and other mammals (Babweteera 2009, Dutton et al. 2014); questions ii–iv were therefore posed so as to better understanding factor contributing to *C. millenii*'s regeneration ecology in Ngel Nyaki. Being one of the larger and most diverse forests in the area (Chapman and Chapman 2001), understanding factors crucial in the regeneration process of its tree populations will elucidate how these threats may impact the long-term survival of this forest.

4.3 Methods

4.3.1 Surveying the recruiting juvenile population

Because seed dispersal is largely leptokurtic (reveiwed in Wunderle 1997) it might be expected that the significant majority of juveniles occur in closer proximity to adult trees. For this reason, it was decided to conduct surveys for juveniles within a 25m annulus of mature adult trees for *C. millenii* and *E. angolense*. A juvenile was defined as anything up to 400 cm tall with a diameter at breast height (DBH, 150cm high) <10cm (*sensu* Babweteera and Brown 2010). For *L. trichilioides*, because of the sheer number of juveniles encountered in the field it was necessary to find a more practical sampling technique (FIGURE 4.2). Around selected adults three radial 25m long transects were extended, each spaced at approximately equal distances apart unless natural obstructions prevented me from doing so. The direction of the first transect was randomized and the last two transects were placed accordingly. Juveniles were recorded in quadrats at 2.5m increments along the transects (e.g. 0–2.5m, 2.5–5m... up to 22.5–25m), and heights and DBH measured as for *C. millenii* and *E. angolense*. The width of the quadrat on either side of the transect increased with increasing distance from the focal tree's trunk so as to account for a greater sampling area at greater distances (*sensu* Matthesius et al. 2011). From 0–12.5m along, width on either side was 1m (i.e. a 2.5×2 quadrat, 5m²); from 12.5–20m along, width on either side was 2m (i.e. a 2.5×4 quadrat, 10m²); and from 20–22.5m along, width on either side was 3m (i.e. a 2.5×6 quadrat, 15m²). Thus the sampling transects were “wedged” shaped

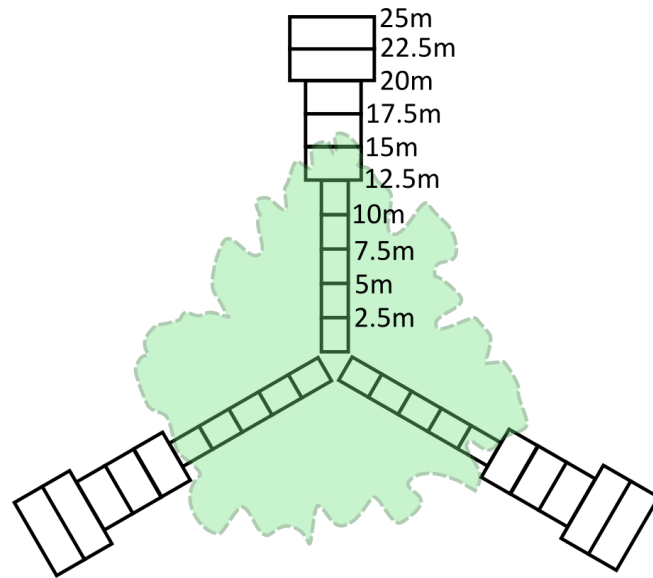


FIGURE 4.2 | The experimental setup for sampling *L. trichilioides* juveniles and *C. millenii* seed dispersal. This (to scale) figure illustrates the three radial transects extended from the trunk of a focal tree and the adjacent quadrats placed at 2.5m intervals (with width increasing at increasing distances). The shaded green area represents that crown of the tree. Crown edge distance from the trunk was estimated to the nearest quadrat interval.

Juveniles were then categorized into different age classes based on their height (a slightly modified system of Babweteera and Brown 2010): 0–50cm tall = seedlings; 51–200cm tall = small saplings; 201–400cm tall = large saplings; and >400cm tall, but with a DBH <10cm = poles. Juveniles found around adult trees were pooled separately for each species and the relative proportions of each class contributing to the entire surveyed juvenile population were calculated. In total 35 *C. millenii*, 12 *E. angolense*, and 10 *L. trichilioides* adult trees were sampled for juveniles. While efforts were made to ensure sampled *E. angolense* and *L. trichilioides* adults were spaced at least 50m apart from each other (but not necessarily other conspecifics) to prevent overlap in survey area, this was not possible for *C. millenii*, which exhibited a highly clumped distribution and exhibited a low number of juveniles (so a heavier sampling effort was required); thus a large amount of the surveyed area for each *C. millenii* adult overlapped with that of other sampled trees.

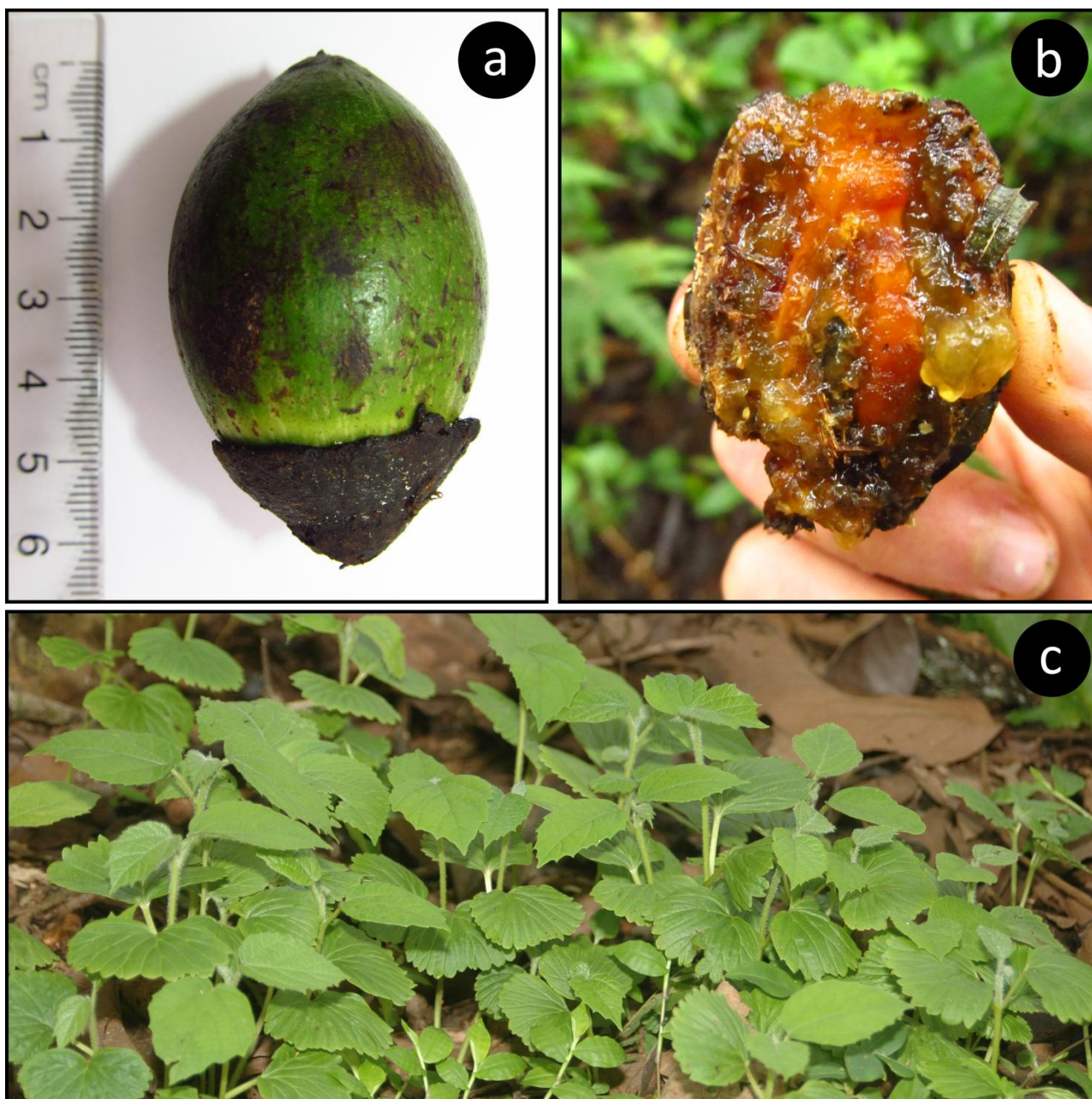


FIGURE 4.3 | *C. millenii* fruit and seedlings. (a) A fruit. (b) A predated fruit, with gnawed epicarp, exposed seed and gelatinous pulp. (c) A cluster of seedlings under the isolated Mayo Kamkam tree. PHOTO CREDIT: Joshua Thia (a, b) and Idris Musa (c).

4.3.2 Measuring seed dispersal profile of Ngel Nyaki's *C. millenii*

As discussed above in SECTION 3.1, seed dispersal is a critical part of the recruitment process. The fruit of *C. millenii* range from ~2.5–3.5cm long, ~1.2–1.8mm wide, and weigh ~2.4g (Keay 1989, Dutton et al. 2014). The single seed is encapsulated in a hard, woody shell, and covered in a gelatinous pulp (personal observation; see FIGURE 4.3). Chapman et al. (1999) in their study in an

East African forest described *C. millenii* as a canopy-level, non-pioneer species, with large seeds. Fruit are initially green, but turn yellow and brown when ripe (personal observation).

Seed dispersal was measured using three 25m long radial transects extending from the trunk of fruiting adults, counting fruit in quadrats every 2.5m, with increasing quadrat width either side of the transect with increasing distance from the trunk (as per FIGURE 4.2). A large amount of whole intact fruits were observed on the forest floor, but also fruit that had been handled by animals, presumably by monkeys like the putty-nosed monkey (*Cerocopithecus nictitans*) that were occasionally seen in the crowns of fruiting individuals, or perhaps ground-dwelling rodents like the *Cricetomys* rat species or the porcupine *Atherurus africanus* that have both been identified as prominent granivores in Ngel Nyaki (Aliyu et al. 2014, Dutton et al. 2014, J. Thia unpub. data). However, because frugivores/granivores often broke seeds into multiple pieces it was not possible to count the number of handled fruit/seed on the forest floor with confidence. Therefore, in each quadrat along each of the three transects only whole, intact fruits were counted; this means that the seed shadow observed is that created by non-animal agents of dispersal, i.e. wind and gravity.

In total three fruiting adult trees were sampled (two in Ngel Nyaki and one in Kurmin Danko), each with three transects. In addition to counting fruits in each quadrat, the crown edge distance to the nearest quadrat (placed along the transect) was measured (i.e. crown edge could be 2.5m, 5m... up to 25m). What was evident when sampling was that not only was crown distance variable amongst trees, but also within a tree; i.e. trees had asymmetric crowns. This meant that dispersal from the trunk can become biased with greater crown spread. For this reason, a simple standardization index (d/c) to account for disparity between the horizontal ground distance from the trunk (d) and the distance that a crown extends from the trunk over the transect (c) was used. For example, if the crown extends ~10m over the transect along the ground, at 2.5m, $d/c = 2.5/10 = 0.25$; ergo, at 5m, $d/c = 0.5$, etc. The d/c value can be essentially interpreted as the “number of crown distances from the trunk”; i.e. a value of 1 indicates the position where the crown ends, a value of 2 represents a distance two times greater than that the crown extends, 0.5 a position half the crown distance, etc. Values for d/c in this data set go up to 10.

The abiotic dispersal of *C. millenii* was illustrated by plotting fruit density as a function of d/c (ground/crown distance). Density of seed per quadrat was calculated by dividing the number of fruit found in the quadrat by the area. All transects were pooled together for the analysis in R v3.1 (Team 2011). A nonlinear least squares (NLS) model was used to model the relationship between density and d/c because the pattern of seed dispersal was blatantly nonlinear and highly left-skewed.

4.3.3 Quantifying the extent of *C. millenii* seed predation

Rates of seed predation can be impacted by habitat fragmentation, increasing in more disturbed environments, and edge habitats may also experience greater rates of predation than interior core forest—both of these may impact seedling recruitment (Jorge and Howe 2009, Aliyu et al. 2014). Because the Ngel Nyaki *C. millenii* population is situated at the edge of the forest, it is possible that seed that falls to the forest floor may exhibit high rates of predation. Additionally, predation rates may be dependent on the abundance of seed, which impacts encounter and detection rates (Blake and Loiselle 1991, Peres et al. 1997).

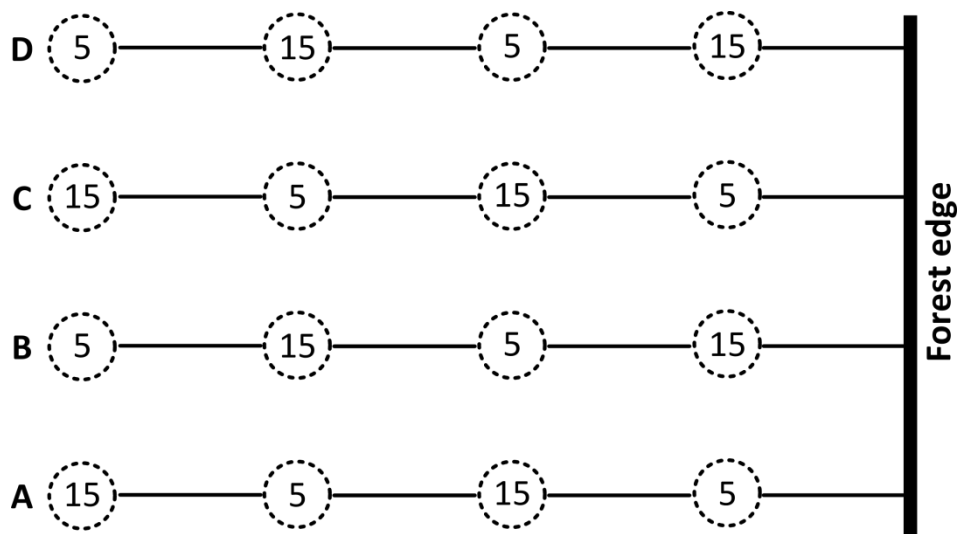


FIGURE 4.4 | Experimental setup for *C. millenii* seed predation experiment (not to scale). Four transects (A–D) were spaced 12.5m apart, each heading into the forest for 80m. Every 20m along these transects a plot (circles) was placed with either five or 15 *C. millenii* fruit.

To assess rates of seed predation, two sites were established (Sites 1 and 2). Site 1 was established on 23 June 2013, and Site 2 on 24 June 2013, which was during the rainy season. A grid-like sampling array with different seed densities was made, see FIGURE 4.4. Each site contained a considerable proportion of the known adult *C. millenii* population in Ngel Nyaki. Per site, four transects (A–D) extending from the edge into the forest and were spaced 12.5m apart. At 20m intervals along transects, a plot was placed, into which *C. millenii* fruit was deposited. This fruit was collected from a single, abundantly fruiting tree (Cor4459) at Kurmin Danko Forest. The first plot was placed 20m in from the forest edge, the next was 40m into the forest interior, the next 60m in, and a final plot at 80m. The number of seeds per plot alternated between five and 15 seeds.

Predation rates in each site were monitored almost daily for the first week, and this gradually became a weekly event as time progressed; in total, each site ran for 57 days. Each time a site was checked, fruit remaining in each plot was counted. Because seed predation was extremely low, it was decided that the data was not worth analyzing.

4.3.4 Understanding factors that impact recruitment in *C. millenii*

During the surveys of juvenile populations, it became evident that *C. millenii* was not regenerating well in Ngel Nyaki Forest. Serendipitously, an adult tree isolated in pasture was discovered—some 3.6km away from Ngel Nyaki Forest near the Mayo Kamkam—with a multitude of seedlings growing underneath the crown (Cor2898; FIGURE 4.3). This was surprising because recruitment was obviously incredibly poor in the forest, yet under this one isolated tree seeds were obviously germinating well, seedlings were surviving, and some had even become considerably established as poles or young trees. These observations lead to the proposition of questions relating to factors that may be limiting survival of *C. millenii* seedlings inside and outside the forest.

Seedlings under Cor2898 were harvested and transported back to the field station where they were temporarily planted in a cool, shaded spot. To disentangle the impact of (1) conspecific proximity effects, (2) vertebrate herbivory, (3) invertebrate herbivory, and (4) habitat, on the survival of *C. millenii* seedlings, a number of different sites were set up to conduct transplant and exclosure experiments. While it may be argued that harvesting all seedlings from under a single parent for transplant experiments is not the most ideal (because all seedlings likely acclimated to similar conditions and are likely highly related) it provided the best opportunity at the time to explore causes of seedling mortality (seeing as seedlings within Ngel Nyaki itself were rare and it takes months for seed to germinate). In fact, Popma and Bongers (1988) in their work with *C. megalantha* chose to use seed sourced from a single adult to reduce genetic variance amongst replicates.

Sites were established early in the week of 5 August 2013. Seedlings were planted and were monitored until 19 August 2013—if they died before this date they were replanted—and all sites were situated more than 200m from each other. To investigate the effect of habitat (shaded forest versus well lit grassland) and the effect that adult conspecifics may have, sites were placed in grassland (four sites) and two types of forest edge habitat: forest edge where adult *C. millenii* were present (five sites) and forest edge where they were absent (four sites). Grassland sites were located within a fenced off section of grassland surrounding the forest, which is protected from cattle and annual burnings by local herders. Within each site two paired plots were established: one plot was

protected using a cage constructed out of wood and wire mesh and the other was left open; this was done to explore the effects of large vertebrate exclusion on seedlings survival. Within each plot, 16 *C. millenii* seedlings were planted in a 4×4 array; each seedling was numbered (based on their position in the array) to ensure they could be tracked through time. Half the seedlings per plot were marked with a small piece of electrical wire, which was used to designate them for invertebrate protection; the other half per plot were left as controls. Invertebrate protection involved the placement of Yates Blitzem snail pellets in ring-shaped indentation around seedlings (ongoing) and spraying with a local pesticide (Rambo) during the dry season. Pesticide could not be applied during the wet season as it needs to be left for at least 24 hours without getting wet, which could not be guaranteed at that time of the year.

After the two week acclimation period (in which seedlings were replaced if they died), recording of mortality began on the 19 August 2013 and plots were monitored approximately once a week thereon (though sometimes the intervals between visits was larger). Death events were recorded as being observed on the day a plot was surveyed. Thus the dataset contains both interval and right censoring (the former indicating an individual dies within some known period but the exact time is unknown, and the latter indicating that the individual was known to be alive by the end of the sampling period). Altogether, 225 days of survival data were used for analysis.

All statistical analysis was conducted in R v3.1.0. Initially, a repeated measures analysis on survival was performed, in which the percent of seedlings alive for a given treatment (the response) was modeled as a function of habitat (HABITAT), vertebrate exclusion (CAGE), and protection against invertebrates (PROTECTION), with time as a random variable (DAYS). Because of the nested nature of the design, an error structure of SITE/CAGE was used. Within each site there were six seedlings for every possible treatment combination.

To affirm the findings of the repeated measures analysis, a survival analysis of between habitat survival rates was conducted using two separate analyses: one where the interval nature of sampling was taken into account and another where only right hand censoring was considered (i.e. the right censoring analysis treats the sampling time as the time of death, as opposed to the possibility that the individuals died sometime between sampling events in interval analysis). The reason for these two different methods was due to the inability of the `survdiff()` function (a function that conducts a Chi-square test of independence on survival between treatments; Harrington and Fleming 1982) to analyze interval data. Regardless, both the interval and right only censoring methods produced similar results.

4.4 Results

The age class structure of the juvenile population in each species was based on the number of observed seedlings found growing within a 25m radius of selected adult trees (FIGURE 4.5). In total, eight *C. millenii*, 142 *E. angolense*, and 2550 *L. trichilioides* juveniles were observed. While the distribution of age classes consisted of >50% seedlings, a large proportion of the juveniles surveyed for *E. angolense* and *L. trichilioides* were also saplings. In comparison, no *C. millenii* saplings were observed and the only two juveniles that were larger than the seedling class were poles (the largest class), suggesting a skew of juvenile ages to the extremes of the distribution.

Abiotic seed dispersal in *C. millenii* appears to be significantly impacted by distance (with most fruit falling within one crown edge distance of the tree) as predicted by the NLS model (FIGURE 4.6). This exponential decay curve was deemed applicable for describing the data because a Shapiro-Wilk test of normality demonstrated the data to be far from normally distributed ($p < 0.001$) and thus nonlinear. Furthermore, because seed removal was almost never observed it is expected that most dispersed seed remains where it is deposited.

The repeated measures of the split plot survival experiment (TABLE 4.1) demonstrated that survival of seedlings was not affected by the exclusion of either vertebrates or invertebrates ($p > 0.05$ for both PROTECTION and CAGE treatments), though the effect of the HABITAT treatment was significant ($p < 0.05$; see FIGURE 4.7). Differences between habitats were confirmed in the survival analyses. The major difference in results between censoring methods in the survival analyses are between ~100–200 days, which corresponds to a period where plots were not surveyed between 23 December 2013 and 3 March 2014. Nevertheless, the same general pattern emerges from the data. Based on the interval and right only censoring models (respectively), median survival times were 39.5 and 43 days in the conspecific edge habitat (95% CI 39.5–39.5 and 43–43), 25 and 29 days in the edge habitat (95% CI 25–25 and 29–29), and 91 and 95 days in the grassland habitat (95% CI 91–102 and 95–109). While complete mortality was observed in the forest edge and conspecific edge habitat, a few seedlings persisted beyond the 225 day study period in the grassland (FIGURE 4.8). Chi-square analysis of the right censored data indicated a significant difference between habitat types on seedling survival ($p < 0.001$).

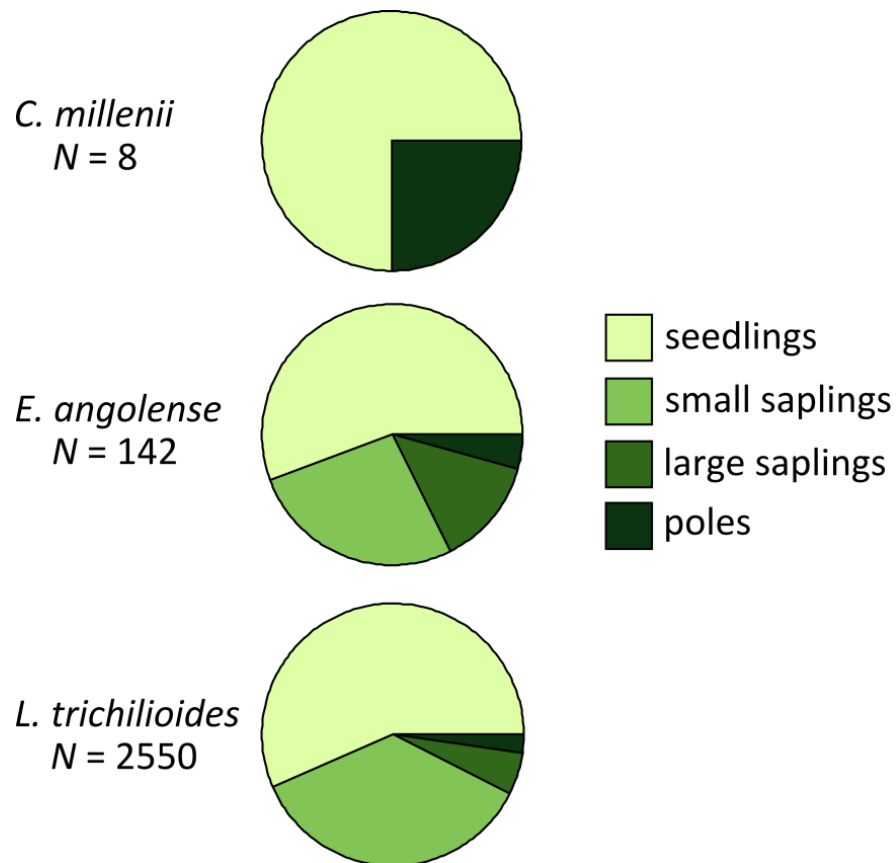


FIGURE 4.5 | Juvenile age structure in *C. millenii*, *E. angolense*, and *L. trichilioides*. *N* = the number of juveniles observed in the field within a 25m annulus of adult trees (see text). Though sampling effort for *C. millenii* (35 adults) was significantly greater than that for *E. angolense* (12 adults) and *L. trichilioides* (10 adults), the number of juveniles observed was dramatically smaller.

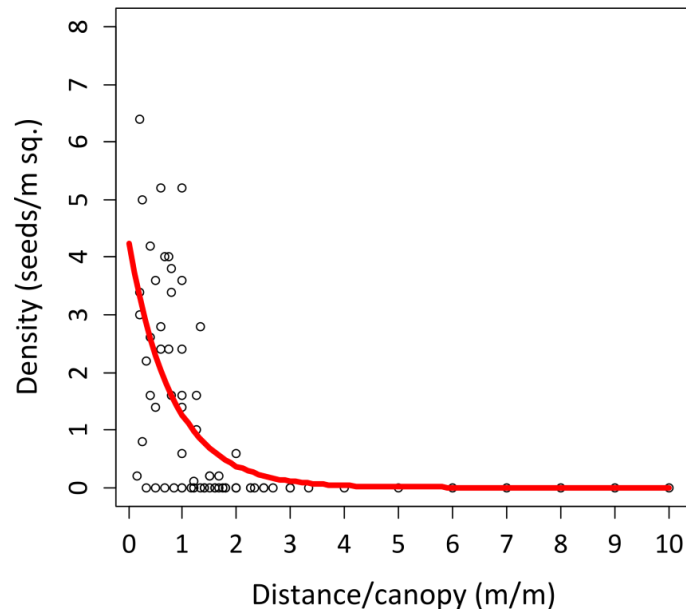


FIGURE 4.6 | Seed dispersal as a function of distance from the parent tree weighted by the crown edge distance. Most seed was observed to fall within one crown edge distance of the tree and seed density rapidly declines beyond one crown edge distance.

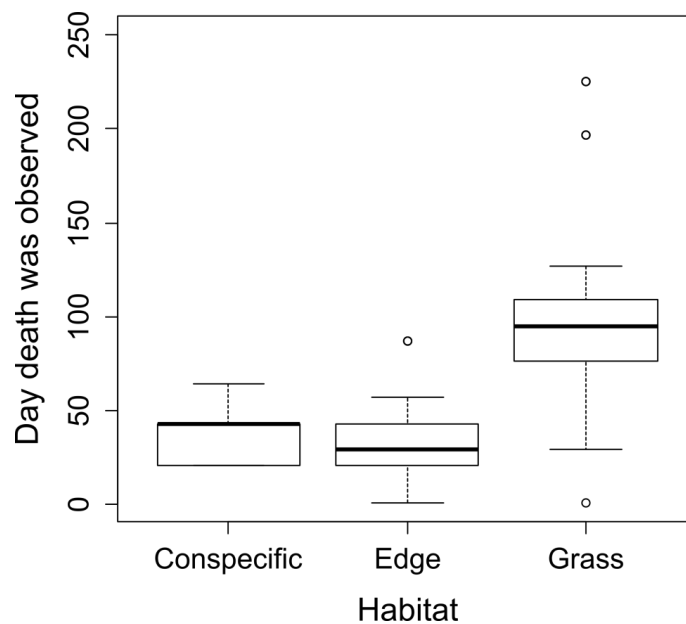


FIGURE 4.7 | Median, upper/lower quartiles, and spread of raw survival data for transplanted seedlings into different habitats. “Conspecific” = adult *C. millenii* trees were present, “Edge” = no conspecific adults, and “Grass” = grassland habitat. The y-axis is indicative of the day at which seedling death was observed (to the nearest sampling event of this study).

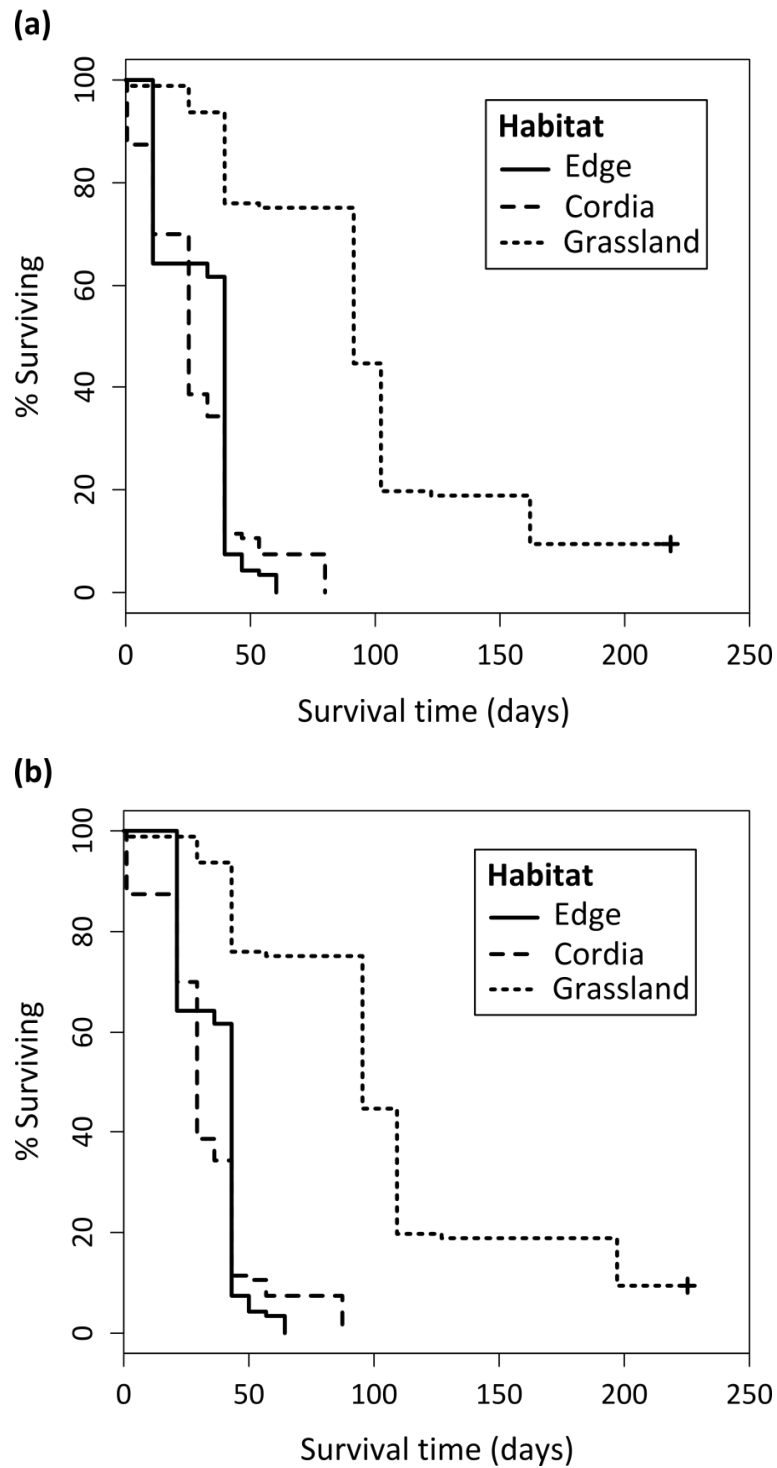


Figure 4.8 | Survival analyses for transplanted *C. millenii* seedlings. Seedlings for all CAGE and PROTECTION treatments in each site were pooled for each HABITAT treatment (edge, conspecific edge, and grassland): (a) results of the interval censoring method, and (b) results of the right censoring only method. The “+” at the end of a line indicates the presence of seedlings surviving till the end of the study period (225 days).

TABLE 4.1 | ANOVA for repeated measures split plot survival experiment. The percentage of surviving seedlings was modeled as function of HABITAT*CAGE*PROTECTION, with DAYS (time) as a random variable, and an error structure of SITE/CAGE.

Error: Site					
	<i>D.f.</i>	<i>Sum. Sq.</i>	<i>Mean Sq.</i>		
Habitat	1	84751	84751		
Error: Site/Cage					
	<i>D.f.</i>	<i>Sum. Sq.</i>	<i>Mean Sq.</i>		
Cage	1	1065	1066		
Habitat*Cage	2	6211	3105		
Error: Within					
	<i>D.f.</i>	<i>Sum. Sq.</i>	<i>Mean Sq.</i>	<i>F-value</i>	<i>Pr(>F)</i>
Habitat	2	188300	94150	69.694	<0.0001
Cage	1	4638	4638	3.433	0.0642
Protection	1	3	3	0.002	0.9630
Habitat*Cage	2	3442	1721	1.274	0.2801
Habitat*Protection	2	285	142	0.105	0.9000
Cage*Protection	1	2201	2201	1.629	0.2021
Habitat*Cage*Protection	2	3766	1883	1.394	0.2485
Residuals	1026	1386029	1351		

4.5 Discussion

The initial objective of this study was to survey and compare the juvenile populations of three tree species in the Afromontane forest fragment Ngel Nyaki for the purpose of understanding how different species might exhibit different patterns of recruitment. Survey results of observed juveniles in the forest suggested that *E. angolense* and *L. trichilioides* are regenerating well in Ngel Nyaki due to the large counts of juveniles and a sizeable presence of older age classes (saplings and poles). Juxtaposed to this is the incredibly small number of *C. millenii* juveniles observed in the forest, along with a highly skewed distribution of age classes toward seedlings and poles (the polar ends of the age class spectrum). These preliminary observations lead to the proposition of questions relating to factors that might be limiting *C. millenii* recruitment in Ngel Nyaki and how anthropogenic disturbance may act as an aggravating force on regeneration of this species.

4.5.1 The importance of primary vertebrate dispersal of *C. millenii* seed

Seed dispersal is a key process in a tree's life cycle because it links the growth and reproductive stages (McConkey et al. 2012) and can facilitate escape from conspecific-related mortality and colonization of microsites suitable for successful recruitment (Schupp 1993, Rey and Alcántara 2000, Jansen and Zuidema 2001, Babweteera et al. 2007, Babweteera and Brown 2010, Effiom et al. 2013). Work on *C. millenii* in Uganda by Babweteera (2009), and Babweteera and Brown (2010), demonstrated that the primary frugivores of *C. millenii* are primates. However, there was a major disparity in the effectiveness of different primates dispersers: small primates never actually ingested seeds and simply spat them out beneath the crown (poor handling), whilst chimpanzees (*Pan troglodytes*) were the most important dispersers because they ingested significant quantities of fruit and provided more effective dispersal services (Babweteera 2009). This pattern is likely applicable to the Ngel Nyaki population of *C. millenii*.

While a lot of fruit fell to the forest floor unhandled, there was also a large amount that was most likely handled by putty-nosed monkeys—which were occasionally seen in the crowns of fruiting *C. millenii*. In this study, the rates of removal of fresh fruit on the forest floor was rare, which is in accord with other studies on *C. millenii* seed removal rates in Ngel Nyaki (Dutton et al. 2014). Therefore, it is with a reasonable amount of certainty that most of the emaciated fruit/seed observed under the crowns of fruiting trees is the product of small primate handling. The hard, woody epicarp of *C. millenii* fruit may limit its attractiveness to rodents due to a potentially higher handling cost (Zhang and Zhang 2008, Vander Wall 2010, Dutton et al. 2014).

Fragmentation and disturbance of forests has been demonstrated to shift the composition of local frugivore assemblages from a community rich in large-bodied and specialist species to a community with a greater proportion of small-bodied and generalist species (Kirika et al. 2008, Babweteera and Brown 2009, 2010). Under such conditions, the dispersal of large-seeded species is likely to collapse, whilst small-seeded species are more likely to retain adequate dispersal and are thus more resilient to defaunation of the frugivore community (Babweteera et al. 2007, Cramer et al. 2007, Babweteera and Brown 2009). The mammalian community of Ngel Nyaki Forest has undergone serious declines in recent decades with the increased presence of human activity in the area (see SECTION 1.4.1). Results of this study reveal an incredibly constrained pattern (a rapid exponential decay) of seed not dispersed by mammalian vectors, and the Ngel Nyaki avian frugivore community lacks species that are able to disperse *C. millenii* seeds (Dutton et al. 2014). However, it must be noted that the amount of replication in this study was limited due to only a fraction of the known adult *C. millenii* population fruiting: only two adults fruited in Ngel Nyaki during the field season and the other replicate came from a tree in Kurmin Danko.

Work in the Neotropical congener *Cordia alliodora* found that seed dispersal is important to escape conspecific proximity mortality and to colonize gaps where survival is greater relative to in the understorey (Augspurger 1984). Additionally, a study on *Cordia bicolor* (also Neotropical) demonstrated declines in seed dispersal with respect to hunting pressure—i.e. due to loss of specialized vertebrate dispersers (Beckman and Muller-Landau 2007). Given that studies elsewhere in Africa show a sensitivity of *C. millenii*'s frugivores to anthropogenic disturbance and fragmentation (Babweteera 2009, Babweteera and Brown 2009, 2010), there is need for concern in regard to the long-term regeneration of this species in Ngel Nyaki. Because the generation time of trees and their dispersers is highly disparate, the effects of large frugivore decline on Ngel Nyaki's *C. millenii* population may not be observed until sometime in the future due to a lagged response (McConkey et al. 2012).

While scatter-hoarding rodents are known to be important vectors of dispersal in the Neotropics (Forget 1990, Jorge and Howe 2009, Jansen et al. 2012) and potentially in the Afrotropics (Nyiramana et al. 2011), including Ngel Nyaki (Aliyu et al. 2014), this study in conjunction with others (Babweteera 2009, Babweteera and Brown 2009, 2010, Dutton et al. 2014) suggests that *C. millenii* benefits little from secondary dispersal and is virtually dependent on primary dispersal. Chimpanzee are believed to be the primary dispersers of *C. millenii* seed (Babweteera 2009), and chimpanzee treatment of seed (through defecation and wadging) has been demonstrated to provide rapid germination in other Afromontane tree species (Gross-Camp and Kaplin 2005). Undoubtedly loss or even continual declines of chimpanzees in Ngel Nyaki from increased anthropogenic stress

will bid poorly for the long-term regeneration of large-seeded, animal-dispersed species like *C. millenii*.

4.5.2 Factors driving the survival of *C. millenii* seedlings in Ngel Nyaki

Causes of mortality to *C. millenii* seedlings in Ngel Nyaki Forest were assessed in a 225 day experiment that explored the importance of habitat, exclusion of large-bodied vertebrates, and protection from invertebrates, on the survival of seedlings. Because so few seedlings were found within Ngel Nyaki Forest itself, all seedlings were sourced from under a single adult tree isolated in grassland. It may seem odd that seedlings would grow so abundantly in the seemingly more “hostile” grassland as opposed to in the forest (FIGURE 4.3), but isolated trees can actually act as foci of regeneration, accumulating seeds and regenerating juveniles under their crown (Guevara et al. 1986, Chimera and Drake 2010, Pizo and Santos 2011).

In this study, the effect of habitat on *C. millenii* seed survival was found to be the only significant cause of mortality in both a repeated measures and survival analysis. Seedlings transplanted in the forest edge (both in the presence and absence of conspecific adults) had significantly lower median survival than that of seedlings transplanted into grassland, based on survival analysis. The survival analysis also estimated a greater significant difference in median survival for seedlings in conspecific edge versus those in edge habitat. This was unexpected because work on the Neotropic congener *C. alliodora* suggested that seedling survival benefits from increasing distance from adult conspecifics (Augspurger 1984). However, it is possible that this result may be biased by a particularly large amount of early seedling deaths in one edge site that may have lead to a downward skew in this treatment’s median survival (and there were points during the study period where the percent of seedlings alive was equivalent amongst edge and conspecific edge sites). From here on, survival will be discussed in terms of seedlings growing in forest (edge/conspecific edge) and grassland.

While the impact of herbivores (vertebrates and invertebrates; Benítez-Malvido and Lemus-Albor 2005, Grogan and Galvão 2006, McKenna and McKenna 2006, Hall 2008) and pathogens (Bagchi et al. 2014) are sure to influence survival of seedlings, for *C. millenii* seedlings in Ngel Nyaki, these factors appear to pale in comparison relative to the overwhelming effect of habitat. Soil type, the availability of light, leaf litter, moisture and the level of forest disturbance are all contributing factors to a seedling’s probability of successful recruitment (Clark et al. 1993, Rey and Alcántara 2000, Hall et al. 2003, Baraloto et al. 2005, Makana and Thomas 2005). For *C. millenii*, it

appears that light is likely one of the primary microhabitat variables that dictates survival (seedlings in the forest edge receives less solar radiation than those in the grassland).

Though sites in the forest sites did not have completely closed canopies, the crowns of trees would have significantly reduced the availability of light to transplanted seedlings in forest sites; grass sites contrastingly were completely unshaded and would have received an appreciably greater level of sun. Further lines of evidence that support the shade intolerance of *C. millenii* are studies by Babweteera (2009) and Babweteera and Brown (2010), that demonstrated *C. millenii* populations in Uganda do not regenerate in the understorey but instead prefer open gaps due to their light-demanding traits. This has also been reiterated by Chapman et al. (1999), who verified increased mortality propensity of *C. millenii* seedlings in the understorey versus in gaps.

Tropical seedlings can exhibit tradeoffs in fitness components in relation to their ability to survive in low light environments versus establishing in gaps (Baraloto et al. 2005). Regeneration of tropical trees is coupled to the interplay between disturbance and light, and the ability for species to partition their niches likely assists the maintenance of biodiversity in tropical systems (Clark et al. 1993, Hall et al. 2003, Baraloto et al. 2005). While not previously considered a pioneer (Chapman et al. 1999, Babweteera 2009) traits of *C. millenii* would suggest that it very much behaves like a pioneer species. According to Brokaw (1985), pioneers are typically found in gaps, which they colonize from seeds. They experience seed dormancy, germinate in response to environmental cues, and are dispersed by animals. The only criteria that *C. millenii* does not satisfy from Brokaw's description of pioneers is the production of abundant small seeds. While seedlings are able to acclimate toward changes in light availability (Popma and Bongers 1988, 1991), it is probable that *C. millenii* seedlings are intolerant to shade.

In this study, none of the sites within the forest could be considered "gaps" per se. While sites were at the forest edge as opposed to dense understorey, it appears that amount of light available to seedlings was not enough to ensure their survival in the forest. All seedlings in forest sites were dead by day 87 (13 November 2013). The time period (August–November) coincides with the peak of the rainy season and the beginning of the dry season (~mid-October/early November), which can still be pretty wet. Given that thick fog can hang over Ngel Nyaki Forest during the wet season (Chapman and Chapman 2001) and significantly block sun light, it is highly probable that life in the forest for seedlings during this period is highly stressful due to lack of solar radiation.

In contrast, seedlings transplanted into the grassland would have had been exposed to significantly more light, which could itself lead to problems regarding dehydration. Many grassland seedlings survived the wet season, but by 225 days (the end of the experiment and near the

conclusion of the dry season), most of these had died. Because none of the seedlings were watered, it seems logical to conclude that one of the limiting factors for *C. millenii* seedlings in the grassland is water during dry periods or stress from excess sun exposure.

4.5.3 Summary remarks

There are many factors that might influence the success and survival of seedlings into reproductive adults, be it environmental variables or the effect of biotic interactions within the community. This study identified *C. millenii* as a poorly regenerating species in Ngel Nyaki Forest, a forest under threat from anthropogenic pressure. The pattern of seed dispersed by abiotic vectors alone displays a very strong leptokurtic distribution: starting off very dense directly under the parental crown but rapidly decaying beyond that crown's edge. As noted elsewhere (Babweteera 2009, Babweteera and Brown 2009, 2010), *C. millenii* is disproportionally dependent on large-bodied vertebrates like chimpanzees to disperse its seeds. The chimpanzee population within Ngel Nyaki is small (estimated <15), but considerably dense, likely due to the fragmented nature of the forests in the region restricting home-range size (Beck and Chapman 2008). Loss of this population will undoubtedly be highly detrimental, not only for *C. millenii*, but also many other large-seeded species within the forest—especially those that cannot rely on secondary seed dispersal.

While prior work only demonstrated an inability to observe seedlings growing in the understorey (Babweteera 2009, Babweteera and Brown 2010), or investigated the survival of transplanted seedlings within forest microhabitats (Chapman et al. 1999), this study looked into understanding the ability of *C. millenii* to colonise grassland habitat outside the forest (but also supported these prior studies by demonstrating the need of seedlings to colonize adequately lit sites). Ergo, dispersal of *C. millenii* seed is vital for sustainability of the population to ensure seed is able to colonize sunny microhabitats in the forest. While seedlings in the grassland obviously survived longer than those in the forest, they still suffered high mortality later in the experiment from desiccation during the dry season. Thus it seems there might be a fine balance between adequate sunlight and shade requirements of *C. millenii* seedlings that prevent them from being too dry and too light deprived—a real “Goldilocks” conundrum—and this can be achieved in the grassland by isolated trees.

The fact that such an abundance of seedlings were discovered under a *C. millenii* adult in grassland illustrates the value of isolated singleton trees as a nexus for regeneration (Guevara et al. 1986, Berens et al. 2008, Chimera and Drake 2010, Pizo and Santos 2011). Because *C. millenii* is obviously capable of successfully recruiting under trees in grassland, efforts in Ngel Nyaki Reserve

could be made to create plots under large established trees growing close to the forest. *C. millenii* seed could be sown or seedlings planted. Establishing fruit bearing trees like *C. millenii* in the grassland would likely increase rates of forest regeneration by encouraging larger vertebrate frugivores to cross the grassland and utilize food sources outside of the main forest (Janzen 1988, Wunderle 1997). This could then lead to greater dispersal of seed from forest into the grassland matrix, which can be a limiting factor in reforestation efforts (Wijdeven and Kuzee 2000, Holl et al. 2000, Castillo and Stevenson 2010).

In practice, such plans to expand the forest area at Ngel Nyaki will likely meet hard opposition due to the agricultural practices of local people. Large-seeded species do not disperse as readily into grassland as it is (Janzen 1988, Wunderle 1997) and survival of such seeds, and resulting seedlings, in the grassland surrounding Ngel Nyaki will be lessened due to heavy cattle presence and the burning of grassland. As a final note, it seems poignant to reflect on the conclusions drawn by Babweteera (2009): Babweteera's work on *C. millenii* in Uganda demonstrated that this species is heavily impacted by anthropogenic disturbance and may be driven to local extinction without human intervention. *C. millenii* unlike the other two species of this study—*E. angolense* and *L. trichilioides*—is not recognized as being of conservation concern by IUCN (IUCN 2013c), yet it is clear from this work that in terms of population regeneration it is the least successful. This highlights the need to consider many species in forest conservation, as some species may slip through the cracks and miss vital management efforts that may bolster their long-term survivability.

CHAPTER 5

Final remarks

The overall aim of this body of work was to address issues that are relevant to the conservation of Afromontane forests, with a particular focus on those in West Africa. Three focal tree species were chosen for this study based on: (i) their abundance and distribution in the study area, (ii) their global conservation status, and (iii) ecology: *Cordia millenii*, *Entandrophragma angolense* and *Lovoa trichilioides* (SECTION 1.4.2). The bulk of research focused on Ngel Nyaki Forest, and other locales in the greater region near Yelwa Village, which is situated on the Mambilla Plateau, south-eastern Nigeria (Chapman and Chapman 2001). The Mambilla Plateau constitutes a belt of montane habitats that run along the Cameroon Highlands that collectively—with other African highland regions—forms what is known as the “Afromonante Archipelago”: an analogy for Africa’s island-like montane areas that share a surprising similarity in floral composition despite their apparent disconnect (White 1978, 1981).

Afromontane forests may have acted as stable refugia during episodes of historical climatic instability (Fjeldså and Lovett 1997). Those of the Cameroon Highlands require urgent protection because they are rich in biodiversity and endemic species but have very little coverage in terms of conservation area (Bergl et al. 2007). The growing anthropogenic pressure and general lack of understanding of the biodiversity and ecology of species in West Africa, relative to other parts of the Afrotropics, demands a greater effort from conservation biologists to understand what taxa there are to protect and how to protect them (Bergl et al. 2007, Norris et al. 2010).

The montane forests of Africa have experienced a history of expansion and contraction in response to changing climate, such that isolation and fragmentation may be an integral part of their history (Hamilton and Taylor 1991, Maley 1996, Eeley et al. 1999, Elenga et al. 2000, Dupont et al. 2001). Population genetic theory states that fragmentation of forest will result in loss of genetic diversity due to drift, yet the ‘Paradox of Forest Fragmentation’ posits that some tree species may respond more positively to fragmentation, such that their ecology may buffer them from the negative effects of reduced genetic variation (Kramer et al. 2008, Bacles and Jump 2011). Studies that investigate population genetic patterns in multiple co-occurring tree species are rare (but see

Stacy et al. 1996, Hardy et al. 2006, Duminil et al. 2010) and there are issues with finding molecular markers that provide directly comparable measurements (Barbará et al. 2007).

In order to make directly comparable observations of genetic diversity in the three focal species cpSSRs were used (CHAPTER 2). These chloroplast SSR loci (ccmp) were developed by Weising and Gardner (1999) as a conserved set of primers that could be amplified across a broad taxonomic range for genetic analyses. Results of this study found that genetic diversity—for the seven ccmp primers used—in all three species was either zero (*C. millenii* and *E. angolense*) or inappreciable (*L. trichilioides*) at Ngel Nyaki Forest. Even across sites in the Yelwa area, and the more distant (~43km away) Akwaizantar Forest, *C. millenii* exhibited no genetic variation—suggesting a trend of genetic homogeneity in the chloroplast lineage for this species on Mambilla. An interpretation of these results is that tree populations on the Mambilla Plateau (in general) have experienced historical drift pre-recent fragmentation, which has resulted in: (1) similar trends of low cpSSR genetic variance across different tree species in Ngel Nyaki; and (2) a homogeneous ccmp haplotype distribution for *C. millenii* between forests. Trends in chloroplast diversity were reflected in DNA sequences of intergenic spacers (CHAPTER 3): *C. millenii* in the Yelwa area were identical to those at Akwaizantar Forest at *trnH-psbA* and *trnL-trnF*. This is not necessarily unexpected given that these forests are still within the same general region.

Because montane habitats are often associated with high biodiversity and a propensity to foster radiations (Fjeldså and Lovett 1997, Roy 1997, Voelker et al. 2010), work was conducted to determine if the *Cordia* species found at Ngel Nyaki and the greater Mambilla region could be considered a discrete taxon (CHAPTER 3). To do so, phylogenetic trees were constructed using a combination of the nuclear ITS1 region and the intergenic chloroplast spacers *trnH-psbA* and *trnL-trnF*. Unfortunately, a clean ITS1 sequence was unable to be obtained from the only specimen of *C. millenii* not from the Mambilla Plateau that I had access to. Even though efforts were made it was also impossible to get a clean sequence of ITS2 from this specimen as a possible surrogate. The cpDNA sequences indicated complete sequence identity between *C. millenii* in the Congo with those found on the Mambilla Plateau in Nigeria. Furthermore, use of seven cpSSR primers to compare inter-regional differentiation found only a single base pair difference in ccmp haplotypes between Mambilla and Congo haplotypes—reinforcing that these populations share similar chloroplast genomes.

While morphological differences lead to speculation of a new taxon, the cpDNA data provides reasonably strong evidence against distinct Mambilla *Cordia* taxa, suggesting that isolation of these montane populations in Nigeria has not been sufficient to accrue significant genetic differences with

those found in other regions. Also, the lack of inter-regional divergence in the cpSSR loci implies high conservatism in the ccmp primers. In hindsight, this could possibly be a contributing factor as to why no haplotypic differences were seen in Ngel Nyaki *C. millenii*—but also *E. angolense*—and explain the minute amount of variation in *L. trichilioides*. Chloroplasts have a much smaller effective population size than nuclear genomes, meaning they can drift to fixation more rapidly (Provan et al. 2001, Charlesworth 2009), and given that cross-amplification of SSR primer sets can often yield fewer polymorphisms (Barbará et al. 2007), this could possibly explain the apparent lack of observable cpSSR haplotype diversity.

This study also wished to address the theory put forward by Gottschling et al. (2005) that all African *Cordia* species originate from a single monophyletic group, the Myxa lineage (CHAPTER 3). While Gottschling et al. (2005) sampled a number of African *Cordia* species, they did not sample any from West African regions. Herbarium samples of *C. africana*, *aurantiaca*, *millenii*, *platythyrsa*, and *senegalensis* were sourced from Kew Royal Botanic Gardens. Sequences of cpDNA *trnH-psbA* and *trnL-trnF* were obtained from all samples and ITS1 from all but a single *C. millenii* sample from the Congo (as discussed above). While sequence variation within a species was not seen in the cpDNA loci, there was variation in the ITS1 sequences. Analysis of sequence data using maximum parsimony and Bayesian inference demonstrated that regardless of loci or method used to construct the phylogeny, the West African *Cordia* species always fell into a single monophyletic group that identified with the Myxa clade observed by Gottschling et al. (2005).

However, the resolution of relationships between the West African species was strongly correlated to the locus used: ITS1 > *trnH-psbA* > *trnL-trnF*. Debate exists around which loci make the best DNA barcodes for plant systematists, a problem that does not really exist for animals due to the relative ease that COI can be used for taxon discrimination (Provan et al. 2001, Pennisi 2007). Initially, the ITS1/*trnH-psbA* combination was chosen because prior studies suggested that it is a good choice for delimiting the relationships amongst species (Kress et al. 2005, Li et al. 2011). The *trnL-trnF* locus was added later to try and add greater resolution to the chloroplast data, but actually proved less variable than *trnH-psbA*. Altogether, phylogenetic analysis supports two major relationships amongst the West African *Cordia* studied: (1) *C. millenii*, *platythyrsa* and *senegalensis* are closely related and form a monophyletic group that split from (2) *C. africana* and *aurantiaca* that form an outgroup to them.

This work demonstrates that the systematic relationships of African *Cordia* are unambiguous and clade assignment within the Cordiaceae could possibly be carried out easily with either chloroplast or nuclear markers—though the clarity of relationships observed will be reliant on the

specific loci selected. Given the loci in this study, ITS1 appears to be the best marker of choice for the Cordiaceae (as in Gottschling et al. 2005), as it was the only marker to show intra-species variance and is more likely to partition out differences between closely related *Cordia*. However, cpDNA loci (particularly *trnH-psbA*) can be used with confidence if DNA is too degraded to successfully amplify useable ITS1 sequences (e.g. in the case of herbarium samples).

As a final part of this thesis, work was carried out to understand the patterns of regeneration in each of the three study tree species (CHAPTER 4). Surveys around adult trees suggest that while regeneration of *L. trichilioides* and *E. angolense* in Ngel Nyaki is occurring, this is not the case for *C. millenii*. Aside from almost no *C. millenii* juveniles observed in the forest, the distribution of age classes was highly skewed towards the polar ends of the spectrum (i.e. seedlings and poles), suggesting that a limited number of seedlings in this population survive long enough to become established as saplings (and even fewer as poles or young trees). In contrast, while greater than half of all *E. angolense* and *L. trichilioides* juveniles were seedlings, there were still a large number of saplings observed around adults.

Experiments were conducted to understand what might be causing arrested recruitment in Ngel Nyaki's *C. millenii* population. Measurements of seed dispersed abiotically demonstrated an inability for seed to disperse far beyond the crown of fruiting trees. While it has been demonstrated that rodents can cause significant mortality to large seeds in Ngel Nyaki (Aliyu et al. 2014), observations of *C. millenii* seed removal suggests they are not favoured by the forest's regular granivores. Transplant experiments demonstrated that light is a major limiting factor to *C. millenii* seedling survival (as suggested by other studies; Chapman et al. 1999, Babweteera 2009): generally, survival of seedlings transplanted in the forest was significantly shorter than those in grassland. While the greater light in the grassland obviously benefits seedlings, conditions become too harsh during the dry season for most seedlings to survive (i.e. the lack of rain, higher temperatures and long hours of sun exposure lead to desiccation of many seedlings). Taken together, these results suggest that *C. millenii* is reliant on seed dispersers to move seed beyond the crown into microhabitats where both light and moisture are sufficient for seedling establishment.

The work presented in this thesis can be used to help understand how to manage fragmented populations of trees on Mambilla, but also in the greater Afromontane region. Based on the cpSSR haplotype data in this study, regional subpopulations might exhibit very little genetic diversity or differentiation between them, indicating fixation of chloroplast lineages. However, this work also highlights the need for SSR markers that are both easily cross-amplifiable but highly resolute, in that they are able to efficiently detect true levels of polymorphism in populations. Future work

could consider the use of nuclear SSRs to provide information on the biparentally inherited genetic elements in these populations; however, this would probably come with the sacrifice of direct comparability between primer sets. Work relating to the movement of pollen between fragments on Mambilla would be highly beneficial to conservation, as it would help understand the relationship between physical and functional connectivity of fragments and populations, which could then be used to direct management.

The *Sporobulus* grassland surrounding Ngel Nyaki is a hostile habitat: not only does it get very parched during the dry season, but the presence of cattle and fire will likely act as an insurmountable barrier to regeneration (Adanu et al. 2010). Even if seeds disperse into the grassland their chances of survival will probably be slim. Already in Ngel Nyaki, fenced off areas have been established around portions of the forest edge. These act as buffers to fire and cattle to help facilitate natural regeneration of tree species in pasture. New directions for reforestation efforts at Ngel Nyaki should look at the use of isolated trees as a point of genesis for new forest fragments (see Guevara et al. 1986, Berens et al. 2008, Chimera and Drake 2010, Pizo and Santos 2011).

As observed in this study, *C. millenii* seedlings were able to recruit under a single parent tree in pasture. The abundance of juveniles and the presence of different cohorts and age classes suggest that, for this species, these microhabitat conditions are highly favourable for juvenile establishment. New priorities should be given to fencing off areas around established trees in close proximity to Ngel Nyaki. This area would be excluded from cattle and a buffer zone could be created around its border to exclude fire. Trees with fruits favourable to frugivores (including *C. millenii*) could then be planted under the shade of the crown so as to develop a new grove that will one day bear many fruiting species to lure frugivores from the main forest. In the long-term, it would be hoped that this will encourage: (a) greater functional connectivity amongst forest fragments; (b) movement of larger-seeded species from Ngel Nyaki into the grassland; and (c) an increase in the forested area at the reserve as forest fragments grow and coalesce.

The broader implications of this study to the conservation of Afromontane trees should be addressed. Fragmented tree populations that have historical contractions in population size and disconnect from each other may exhibit low levels of genetic diversity, especially in the chloroplast, which suffers particularly from bottlenecks. Little can probably be done to remediate this, as this phenomenon is the product of natural historic processes. Instead, efforts should be made to understand where genetic variance is partitioned amongst populations and how anthropogenic pressure may inhibit gene exchange across the landscape. This work also demonstrates that species not globally recognized as being of conservational concern (e.g. *C. millenii*; see Babweteera 2009)

can be highly susceptible to habitat disturbance, requiring more attention than more supposed “at risk” species. Ergo, those responsible for prescribing conservation actions should first assess what species are in the most jeopardy at a local level (as these could be overlooked and lost without proper foresight).

This present study applied a combination of genetics, systematics and ecological work to contribute to a greater understanding of processes that have shaped, and continue to shape, Ngel Nyaki Forest and other forests on the Mambilla Plateau. In conclusion, conservation of the montane forests of Africa will require substantial support from conservation scientists, local people, and government bodies to help balance the needs between people and the environment. Forests like Ngel Nyaki represent relict communities that are diverse in flora and fauna and thus deserving of greater conservation efforts than they currently receive. As forests disappear across Africa such isolated montane forests may represent the last stand for some species in their region, and montane forests may also act as refugia during periods of climate change. Thus with urgency, conservationists need hasten to make sure Afromontane forests have a secure future.

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